Trop J Nat Prod Res, January 2024; 8(1):6028-6034 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Anti-SARS-CoV-2 Activity and Acute Toxicity Screening of *Annona muricata* and *Artemisia annua* Leaf Extracts

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ARTICLE INFO

Article history:
Received 04 September 2023
Revised 13 December 2023
Accepted 02 January 2024
Published online 01 February 2024

ABSTRACT

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Available data suggests that there is no specific treatment in modern medicine. The present study aims to investigate the anti-SARS-CoV-2 activity of *Annona muricata* and *Artemisia annua* leaf extracts as well as their acute toxicity study.

The phytochemical screening of the plant extracts was done using standard methods. The acute toxicity evaluation was done using Lorke method. The antiviral activity screening against SARS-CoV-2 was done by plaque assay with Vero-E6 cell line.

Phytochemical screening revealed the presence of alkaloids, terpenoids, flavonoids, saponins and steroids in both plants, while tannins and anthraquinone glycosides were absent. The quantification of SARS-CoV-2 by plaque assay with Vero (E6) cell line shows a reduction in the zones of cellular death for the extracts treated cells compared to the cells with the virus only. Acute toxicity test showed no death or abnormal behavioural changes in mice during and after 24 hours of extract administration.

On the basis of the results obtained from the present study, both *Annona muricata* and *Artemisia annua* leaf extracts are considered to have some level of inhibition against SARS-CoV-2 virus growth.

Keywords: Medicinal plants, Phytochemicals, COVID-19, SARS-CoV-2, Antiviral activity, Toxicity.

Introduction

The coronavirus disease 2019 (COVID-19), resulted in an outbreak of pathogenic viral pneumonia in Wuhan, Hubei Province, China, in December 2019.[1] The subsequent spread has led to a global pandemic.[2] COVID-19 disease appears to be a spectrum of clinical presentations ranging from asymptomatic to severe respiratory failure.[3] Initial case analysis from China through mid-February 2020 found 14% of cases were associated with severe disease and 5% of cases were critical (i.e., respiratory failure, septic shock, and/or multiple organ dysfunction or failure).[3] A more extensive meta-analysis found a slightly higher severe disease percentage (20.3%).[4] The disease case fatality rate (CFR) varies depending on region, population demographics, and healthcare capabilities.[5] The management of the disease condition using known and established orthodox drugs are basically to provide supportive therapy.

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Citation: Erharuyi O, Aghahowa S, Igbinosa E, Imieje VO, Ogbeide OK, Akhigbe IU, Nahandoo I, Olowoeyo BE, Akubuiro PC, Ayorinde J, Igoli JO, Falodun A. Anti-SARS-CoV-2 Activity and Acute Toxicity Screening of *Annona muricata* and *Artemisia annua* Leaf Extracts. Trop J Nat Prod Res. 2024; 8(1):6028-6034. http://www.doi.org/10.26538/tjnpr/v8i1.45

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Some drugs like hydroxychloroquine, azithromycin and remdesivir have been used to treat the disease.[6] Some of these antiviral agents might produce toxic side-effects. The development of viral resistance toward antiviral agents enhances the need for new effective compounds against viral infections. Thus, new antiviral agents exhibiting different mechanisms of action are urgently needed.

The use of natural products including medicinal plants has become more and more important in primary healthcare especially in developing countries.[7] Millions of people use herbal medicine for the management of disease conditions. The World Health Organisation supports the use of traditional medicine for the management of diseases provided they are proven to be efficacious and safe.[8] It has been observed that the use of traditional herbal medicines, especially in the tropics and African countries for the management of COVID-19, is on the increase (the case of Madagascar). Therefore, it is necessary to look inwards for the search of novel and effective herbal medicine for the effective management of COVID-19, cannot be over-emphasized.

Natural products serve as chemical scaffolds for derivatization to come up with novel compounds with improved pharmacological features.[9] Surveys of the National Cancer Institute, USA, repeatedly demonstrated that three-quarters of drugs for all diseases worldwide during the past half-century were in one way or another based on natural resources.[9] Hence, chemical scaffolds from natural sources are indispensable for drug development. Nature provides a vast library of chemicals to explore and develop drugs for the treatment of various ailments including viral diseases.[10] To date, a good number of herbal medicines or their constituents have shown potential antiviral

activity.[11] However, there is a lack of adequate research on the development of anti-coronaviruses agents from such natural products. Such agents are not only important to combat coronaviruses, but also play an important role to prevent a viral attack. Some natural products have been found to exhibit their antiviral activity through the inhibition of viral replication.[12,13] Some plants e.g. *Annona muricata*, *Persea americana*, *Lycoris radiate*, *Azadirachta indica*, *Macaranga barteri* and *Andrographis paniculata* have been reported to have inhibitory activity against several pathogenic viruses, including other respiratory viral infections.[14-18] Also, several individual essential oil components of Garlic (*Allium sativum*), Ginger (*Zingibber officinale*) have been screened for antiviral activity.[18, 19] Recently, *Annona muricata* leaf has proven useful in the successful management of COVID-19 and associated symptoms.

Annona muricata Linn. (Annonaceae) commonly known as "Soursop" is a medicinal plant used in many tropical countries. In traditional medicine, various parts of the plant are used in the treatment of various diseases, such as fever, respiratory diseases, parasitic infections, diabetes, hypertension, stomach pain, bronchitis and cancer.[20-25] *Annona muricata* and its acetogenins (a major component of the annonaceae family) have been found to have anticancer, anti-inflammatory, antibacterial and antiviral activities.[22,26,27]

Artemisia annua is a well-known medicinal plant belonging to the Asteraceae family.[28,29] The identification of the plant and its artemisinin component constituted a major breakthrough in the fight against malaria.[28] *Artemisia annua* and it artemisinin component have been shown to have other pharmacological activities such as analgesic, anti-inflammatory, antioxidant, immunomodulatory, antibacterial, anticancer and antiviral activities.[30,31]

The present study is therefore aimed at investigating the antiviral activity of the medicinal plants; *Annona muricata* and *Artemisia annua* against the SARS-CoV-2 virus.

Materials and Methods

Plant collection, authentication and drying

The plant material; *Annona muricata* leaves were collected in February, 2021 from Ekosodin community, Ovia North East Local Government Area, Edo State, Nigeria, while *Artemisia annua* leaves were collected in April, 2021 from Iwajowa area, Ibafo, Ogun state, Nigeria. The plant materials were identified and authenticated at the University of Benin herbarium, and the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria with the following voucher specimen numbers; UBH-A356 and FHI113652 for *Annona muricata* and *Artemisia annua*, respectively. The plant materials were air-dried, powdered and stored in an air-tight container until ready for use.

Phytochemical screening

Simple chemical tests to detect the presence of secondary metabolites such as alkaloids, tannins, saponins, terpenoids, steroids, anthraquinones, flavonoids and other phenolic compounds were done according to standard methods.[32,33]

Extraction

The powdered plant materials (200 g each) were extracted separately by maceration in methanol (2 L) at room temperature for 7 days. The extracts were concentrated using rotary evaporator at reduced pressure.

Toxicity Screening

Experimental animals

Adult Swiss albino mice of either sex were obtained from the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin. The animals were kept under a 12-hour light/dark cycle in clean and well-ventillated cages for two weeks to acclimatize to the laboratory environment. The animals were fed with standard rodent pellets, and allowed access to water *ad libitum*.

Ethical consideration

The animals were handled in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU[34] for animal experiments. Ethical approval was obtained from the Faculty of Pharmacy, University of Benin Ethical Committee with the approval number EC/FP/020/12.

Acute toxicity screening

The acute toxicity screening was carried out according to Lorke method.[35] Nine adult mice of either sex divided into three groups of three animals per group were used in the first phase. The plants' extracts were administered orally at doses of 10, 100, and 1000 mg/kg to groups I, II, and III, respectively. In the second phase of the study, another three mice divided into three groups of one mouse each were used. The plants' extracts were administered at doses of 1600, 2900, and 5000 mg/kg to each group, respectively. General symptoms of toxicity and mortality in each group were observed within 24 hours and then for another 14 days for delayed toxicity.

Evaluation of antiviral activity

Cell lines and culture

Cell lines (Vero E6 cells) were obtained from American Type Culture Collection (ATCC). The medium, phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), and Trypsin-EDTA were brought to room temperature to warm up and to thaw, respectively prior to use. A vial of Vero E6 cells with passage number P21, was brought out of the -80°C freezer, wiped with 70% ethanol and placed inside a BL2 Biosafety cabinet and was allowed to thaw. Cells were washed by adding DMEM with 10% FBS and centrifuged at 400 x g at 25°C for 20 min, after which the supernatant was carefully removed and placed inside a liquid discard bottle. The cell pellet was re-suspended and transferred into 10 mL of 10% DMEM in a T75 flask and was then incubated in a 5% CO_2 incubator at 37°C for 48 h. The flask was then observed for cell growth, cell degeneration, pH, and turbidity under an inverted microscope. After the cells became 80-90% confluent, sub-culturing was done.

Cell seeding

Media from the culture flask was carefully aspirated using a serological pipette. The cells were washed twice with appropriate volume of Phosphate buffered saline (PBS) to remove remnant Serum (Fetal bovine serum) and dead cells. Trypsin (1 mL) was added to the cells in the flask and swirled. The cells were incubated in a CO_2 incubator at 37°C for 4 min. The sides of the flask were tapped gently to dislodge the cells from the surface of the flask. Growth media (4 mL of 10%) was added to inactivate trypsin and was swirled and triturated 5-8 times to ensure that the cells are in single sub-cellular cells. The cell suspension was carefully aspirated using a serological pipette into a 15 mL falcon tube and was centrifuge at 400 x g, at 20°C for 10 min. The supernatant was carefully aspirated and placed on a discard bottle remaining the cell pellet. The cell pellet was adjusted to 1 mL of cell suspension and was triturated very well to generate a homogenous cell suspension. The mixture was then incubated at room temperature for 2-3 min. The mixture (20 µL) was transferred to the H-shape wells of the hemocytometer with a glass slide fixed on it. The hemocytometer was placed on an inverted microscope at 10x objective lens, a hand tally counter was used to count live cells in each 4 corners of the 16 square boxes of the hemocytometer. Live cells were counted and calculated as follows:

Viable cell = Average of live cells counted x 5(dilution factor) x 10^4(cell counting standard per mL)

The cell suspension (1.5 mL) was taken and 10% DMEM was used to make it up to 12 mL to bring the cell concentration to 750,000 cells/mL, which is appropriate for plaque assay using 6-well plate according to cell seeding guidelines. The cells were thoroughly mixed to ensure even distribution of cells and 2 mL was seeded into each well of the 6-well plate and incubated for 12-18 h at 37°C and 5% CO_2. The Vero E6 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS). Incubation was carried out at 37°C with a 5% CO_2 humidified environment. After 1incubation, the cells were observed under the inverted microscope at 40 x magnification.[36]

Plaque Assay

Approximately 7.5 x 10^5 Vero E6 cells/well were seeded into 6-well plates and incubated overnight (12-18 h) at 37^{O}C, and 5% CO_2. The tittered infectious sample was prepared in 10-fold dilution series (10^{-3} and 10^{-4}) enough volume was provided to make up to 200 µL per well. Existing cell culture media were removed from 6-well plate, 200 µL of each of 10^{-3} and 10^{-4} dilution was added to the well with virus only, whereas the well containing 10^{-3} dilution with *Annona muricata* crude extract (AMCE) and *Artemisia annua* crude extract (AACE) extract was mixed at 1:1 ratio before inoculation but as for the 10^{-4} dilution with AMCE and AACE extracts, 100 µL of 10^{-4} virus stock was first inoculated for 30 min before equal volume of the AMCE and AACE extract was added. The plates were incubated at 37^{O}C in 5% CO_2 for an hour and the plates were rocked intermittently at interval of 15 min to prevent cell from drying out. 2X Minimum Essential Medium (MEM) + 4% FBS with 2% methylcellulose was mixed in a 1:1 ratio and placed in the incubator at 37^{O}C while Incubating the plate to decrease the viscosity of the solution. After 1 h incubation 2 mL of methylcellulose mixture was added to each well of the 6-well plates. The plates were then incubated at 37^{O}C in 5% CO_2 for 3 days. On day 3, methylcellulose overlay was carefully removed with a pipette and the cell was fixed by adding 3 mL of 4% paraformaldehyde (PFA) in PBS to each well and the plate was incubated for 30 min. The 4% paraformaldehyde was removed gently into a hazardous liquid waste container. Crystal violet (1 mL of 0.05%) in 20% methanol was added to each well and was incubated for 30 min. Crystal violet was removed with pipette and each well were washed three times with sterile water and excess crystal violet was removed and plaque was easily visualized under the microscope and the photomicrographs were taken.[36]

Results and Discussion

Phytochemical constituents

In the present study, the extracts from the leaves of *Annona muricata* and *Artemisia annua* were screened for their secondary metabolites and acute oral toxicity as a first step in their antiviral activity screening against SARS-CoV-2. As shown in Table 1, both plants have similar secondary metabolites. Alkaloids, terpenoids, steroids, flavonoids and saponins were found in the leaves extracts of *Annona muricata* and *Artemisia annua*, while tannins and anthraquinones were absent in both extracts (Table 1).

Acute toxicity profile

In the acute toxicity studies using the Lorke model, there were no abnormal behavioral changes, no sign of toxicity and no death occurred during and after 24 hours of the test (Table 2). The results obtained showed that both extracts had LD_{50} above 5000 mg/kg. This suggests that the leaf extracts of both plants are non-toxic, and are relatively safe when consumed. Although, the toxicological profile of *Annona muricata* and *Artemisia annua* have been widely reported, this study sought to validate these claims and also to serve as a preliminary report on the basis of which further toxicological screening will be carried out. While some of the previous findings such as that of Sherif *et al*. (2017)[37] and Al-Medhtiy *et al*. (2022)[38] show that the LD_{50} of *Annona muricata* leaf extract is greater than 5000 mg/kg which corroborated our findings, others observed lower LD_{50} values for *Annona muricata* leaf extract. For example, the work of Agu *et al*. (2017)[39] has shown the LD_{50} of *A. muricata* methanol leaf extract to be 1918.33 mg/kg in rats, the work of Omoja *et al*. (2014)[40] showed an oral LD_{50} of 354.8 ± 8 mg/kg, while that of Aphonse *et al*. (2017)[41] reported oral LD_{50} of 3750 mg/kg. On the other hand, for *Artemisia annua*, the findings from this work in terms of the oral acute toxicity corroborated most of the previous researches such as that of Siddiqui *et al*. (2018),[42] and XuMei *et al*., (2018)[43] which showed the plant to be safe with oral LD_{50} above 5000 mg/kg, while a study reported lower LD_{50} of 2750 mg/kg in mice.[44] The variation in the observed LD_{50} values for both plants could be attributed to differences in the geographical location, stage of maturity of the plants which may influence the type and amount of phytoconstituents present.

Anti-SARS-CoV-2 Activity

The quantification of SARS-CoV-2 by plaque assay with Vero-E6 showed the following results: Figure 1; *Anonna muricata* crude extract (AMCE) was inoculated in two viral titers 10^{-3} and 10^{-4}. In both titres, there were more zones of cellular death in wells with only virus than there were in the wells with both extract and virus. The same effect was also seen in Figure 2 for *Artemisia annua* crude extract (AACE).

The global pandemic of COVID-19 caused by the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) has affected millions of people in several countries around the world. Since its outbreak in 2019 through 2020, a lot of efforts have been geared towards prevention and finding effective treatment for the infection. To this end, many vaccines and therapeutic agents have been developed to mitigate the disease.[45-48]

Table 1: Phytochemical constituents of the plants leaves

Phytochemicals	Inference	
	Annona muricata	***Artemisia annua***
Alkaloids	+	+
Saponins	+	+
Tannins	-	-
Terpenoids	+	+
Flavonoids	+	+
Phenolics	+	+
Steriods	+	+
Anthraquinones	-	-
Carbohydrates	+	+
Reducing sugars	+	+

Present (+)/Absent (-)

Table 2: Acute toxicity profile of *Annona muricata* and *Artemisia annua* leaf extracts

Dose (mg/kg)	Number of mice	No of Death recorded	
Phase 1		***Annona muricata***	***Artemisia annua***
10	3	0/3	0/3
100	3	0/3	0/3
1000	3	0/3	0/3
Phase 2			
1600	1	0/1	0/1
2900	1	0/1	0/1
5000	1	0/1	0/1

Traditional medicines in the form of herbal extracts have also played a role in the prevention and treatment of COVID-19. For example, herbal teas from *Artemisia annua* and *Annona muricata* leaves have been used to prevent and treat COVID-19.

Annona muricata and *Artemisia annua* extracts have been reported to possess antiviral activity against a number of viruses such as human immunodeficiency virus, herpes simplex virus, and dengue virus.[49-52] Annonaceous Acetogenins such as annomuricin a, annomuricin b, annomuricin c, muricatocin c, muricatacin, cis-annonacin, annonacin-10-one, cis-goniothalamicin, arianacin, and javoricin from *Annona muricata* have been shown to have inhibitory activity against SARS-CoV-2 spike proteins *in silico*.[53] Also, phytocompounds from *Artemisia annua* such as arteminisinin, artesunate, artemether, artenimol, artenuin B, and artelinic acid have been shown to possess SARS-CoV-2/host target protein inhibitory activity *in silico*.[54] *Artemisia annua* extract and it phytochemical artemisinin have also been found to possess anti-SARS-CoV-2 activity *in vitro*.[55] To substantiate their potential for use as Covid-19 phytotherapeutic agents, in the present study, we carried out the anti-SARS-CoV-2 activity of the methanol extracts of the leaves of the two plants *in vitro* using the plaque assay.

The plaque assay is the gold standard test for quantifying infectious viruses in a sample. The plaque assay measures ''plaques'' which describe the zone of cellular death that occur after one infectious agent have entered into a cell and spread to adjacent cells over the period of incubation.[36] The assay does not rely on the use of any virus-specific reagents, which is beneficial when reagents are unavailable.[36] As this cell-base assay is typically performed in 6-well plates, it is relatively low-throughput, labour-intensive, and may not be reliable when samples themselves are cytotoxic (e.g. homogenate from certain tissue) or when the virus is poorly cytopathic in a given cell type, it is important to choose a permissive cell type (e.g. Vero-E6 cells) for which SARS-CoV-2 causes substantive cell death.[36]

Due to the high sensitivity of plaque assay with respect to high concentration of cells seeded, any slightest cell distortion and death can be observed which shows that virus indeed invaded the cells.

Conclusion

Based upon the observed results and the assay chosen, both AMCE and AACE extracts are considered to have some level of effect on SARS-CoV-2 virus growth inhibition. Although, the effect was observed to be more when both the virus and the extracts were administered at the same time than when administered thirty minutes after infection. The acute toxicity studies shows that both extracts are relatively safe when administered orally, although, further studies is needed to examine the effects on vital organs. These observations shows the potential of the plants as source of therapeutic agents against SARS-CoV-2 and related viruses.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

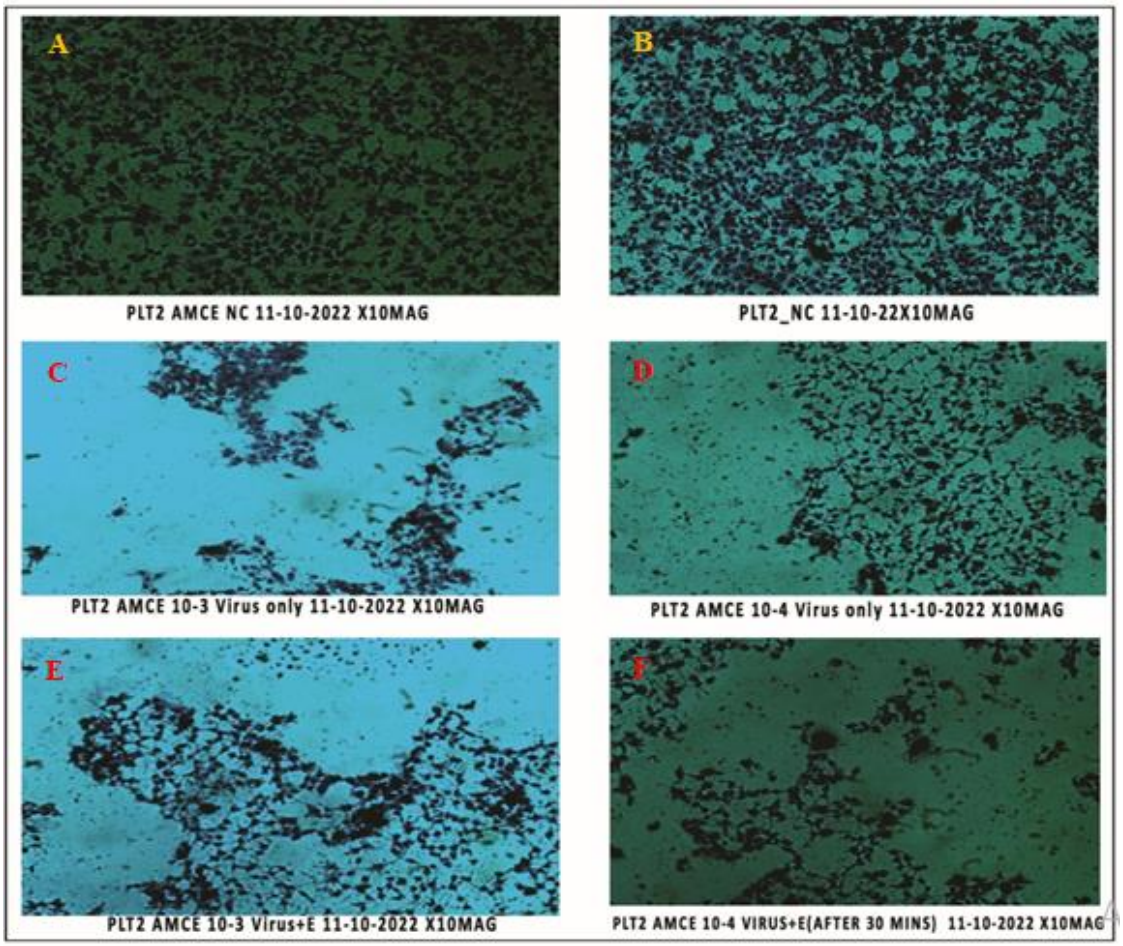

Figure 1: Photomicrographs showing Anti-SARS-Cov-2 Activity of *Annona muricata* crude extract (AMCE) **A & B:** Negative control (Vero E6 only) **C & D:** Virus control (Vero E6 + Virus, at 10^{-3} and10^{-4} dilution, respectively) **E & F:** Test (Vero E6 + Virus + Extract, at 10^{-3} and10^{-4} dilution, respectively).

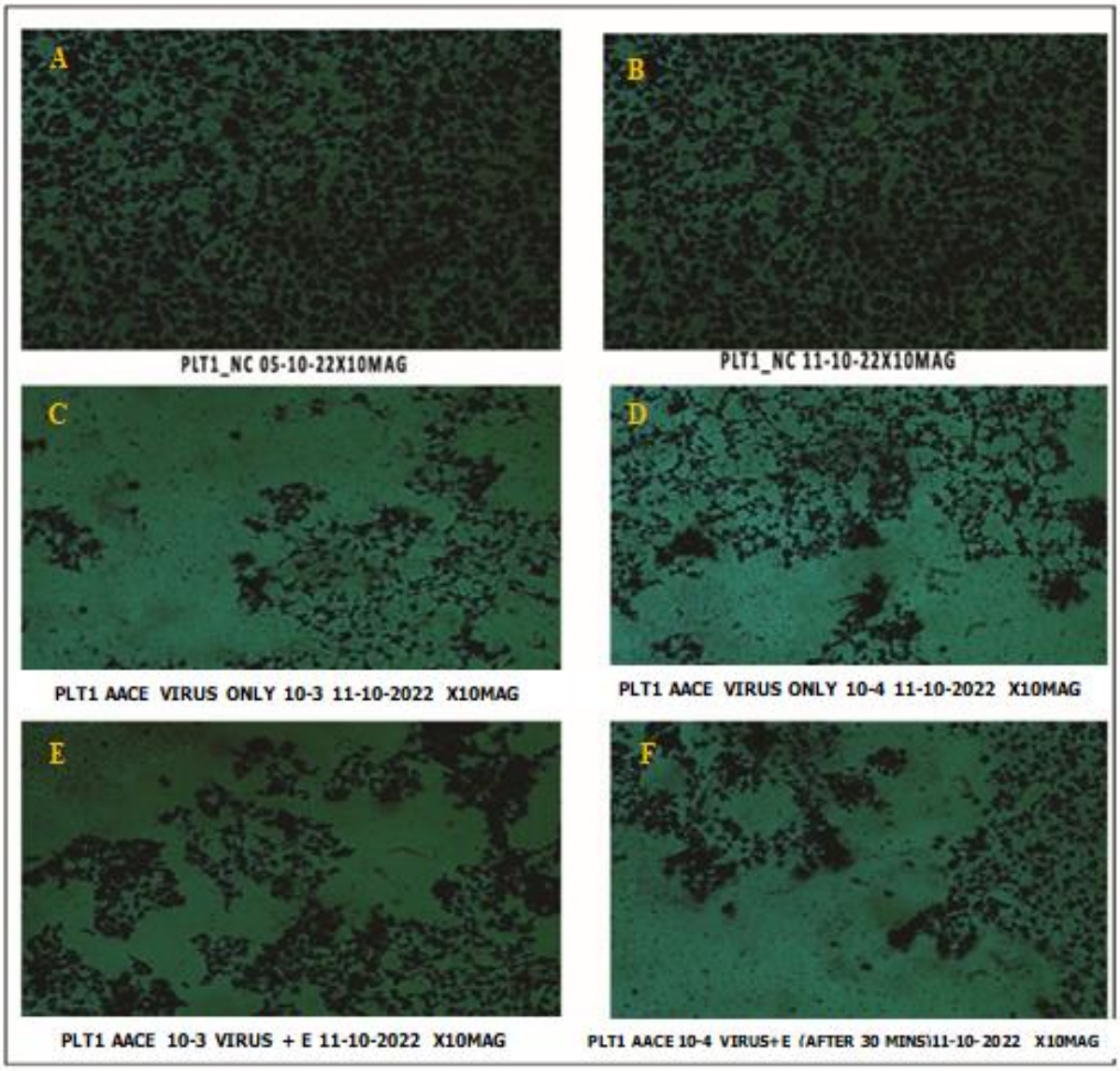

Figure 2: Photomicrographs showing Anti-SARS-Cov-2 Activity of *Artemisia annua* crude extract (AACE) **A & B:** Negative control (Vero E6 only) **C & D:** Virus control (Vero E6 + Virus, at 10^{-3} and10^{-4} dilution, respectively) **E & F:** Test (Vero E6 + Virus + Extract, at 10^{-3} and10^{-4} dilution, respectively).

Acknowledgements

This work is supported by Tertiary Education Trust Fund (TETFund) National Research Fund (NRF) (TETF/ES/DR&D/CE/NRF2020/SETI/56/VOL.1).

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