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Novel dietary herbal preparations with inhibitory activities against multiple SARS-CoV-2 targets: A multidisciplinary investigation into antiviral activities

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ABSTRACT

The outbreak of the COVID-19 pandemic has resulted in not <7.1million deaths globally as of December 2024. Many new variants of concern have continued to emerge since the initial outbreak of the original SARS-CoV-2 virus traceable to the Wuhan strain (Wuhan-Hu-1). In this work, the therapeutic potentials of four new polyherbal dietary preparations – VIVE (five plants), FORTE1(fortified VIVE), COMBI-5 (five spices) and MOK (Moringa seed) as well as four individual ethnomedicinal plants were investigated. Computational screening revealed chemical structures capable of establishing moderate to strong interaction with SARS-CoV-2's main protease enzyme, while in vitro screening against the viral protease clearly established inhibitory potencies. The individual plant extracts making up VIVE and FORTE1 showed mild (494.9 ± 19.6 μg/ml) to moderate (21.5 ± 1.1 μg/ml) inhibitory activity against the viral enzyme *in vitro*; highest activity was obtained in the polyherbal VIVE preparation (17.3 ± 1.4 μg/ml). The MOK exerted total inhibition – 100 % (IC_{50} -3.6 ± 0.9) of the viral enzyme while COMBI-5 produced an inhibition of 95 %(IC_{50} - 0.9 ± 0.1). These results revealed the potential of

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specialized metabolites within these widely consumed dietary herbal products for the management of COVID-19 and related viral threats.

1. Introduction

The COVID-19 pandemic has had a devastating global impact, with about 0.8 billion cases reported and tragically claiming over 7.1 million lives as of December 2024, according to the WHO (WHO, 2024). The emergence of numerous variants of concern since the initial outbreak of the original SARS-CoV-2 virus, traceable to the Wuhan strain (Wuhan-Hu-1), continues to pose significant challenges to global health efforts. In the last few decades, SARS-CoV-2 and two major groups of β-coronaviruses, the severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), have caused enormous global fatalities (Mann et al., 2020; Tarique et al., 2021). SARS-CoV-2 is transmitted by the release of droplets and virus particles by an infected person while breathing, talking, coughing or sneezing especially in a crowded or a poorly-ventilated area. Symptoms like difficulty in breathing, dry cough, fever, chest pain, and sometimes, loss of smell and taste. Preventive measures include social distancing, wearing of face mask, use of alcoholic hand sanitisers, hand washing and partial or total lockdown as community containments (WHO, 2021). Coronaviruses derive the name from the crown-shaped appearance of the virus which is as a result of the large spike glycoprotein, forming extended homotrimers. This spike protein binds to the host cell in animals by interacting with specific proteins such as the aminopeptidase or the angiotensin-converting enzyme II (ACE2). This binding of the spike (S) protein facilitates viral entry into the host cell and the release of the viral genome. Current reports have shown that during a SARS infection, the receptor binding domain of the S protein gets directly attached to the ACE2, which is majorly expressed in the lungs, kidney and intestine, thereby making these organs very vulnerable (Qi et al., 2021). In addition, the presence of type II transmembrane serine protease (TMPRSS2) at the outer membrane of some epithelial tissues such as respiratory, urogenital and gastrointestinal induces proteolytic cleavage of viral spike protein further increasing the viral susceptibility of these organs. A key protein, the main protease (M^{pro}) known as 3-chymotrypsin-like protease ($3CL^{pro}$), is required for the proteolytic maturation of the virus (Gyebi et al., 2021; ul Qamar et al., 2020). Consequently, targeting M^{pro} can provide an effective treatment against SARS-CoV-2 by inhibition of the viral polypeptide cleavage. Furthermore, since this well characterised protein is absent in humans, it forms a very good target for drug discovery. Other drug targets include: papain-like protease (PL^{pro}), RNA-dependent RNA polymerase (RdRp), angiotensin-converting enzyme II (ACE2), SARS-CoV helicase and Type II Transmembrane Serine Protease (TMPRSS2) (Wu et al., 2021).

Although a number of vaccines have been approved, the search for management therapy must be sustained especially from plants, which form a reservoir for bioactive compounds. Furthermore, around 80 % of the global population use plants at one point or the other as part of their primary health care need. Medicinal plants have been used against coronaviruses in the Orient. This is majorly inspired by the success or the beneficial effect in the use of numerous herbal formulas, either as single agents or in combination with conventional therapy especially in China and in the Middle East (Sun et al., 2018).

Furthermore, immune-modulatory agents, which are compounds that are non-specific and exert their activities without antigenic specificity, have been utilised in traditional medicine for the treatment/prevention of coronavirus. Immune boosters act by optimizing body immunity to pathogenic factors, balancing the immune response, reducing hyper-inflammatory states, and fostering body repair. For example, *Vernonia amygdalina*- enhances immune response by increasing the levels of white blood cells and $CD4^+$ (Momoh et al., 2012); also, *Azadirachta indica* (Neem)-derived compounds nimbolin A, nimocin, and cycloartanols have the potential to bind to some SARS-CoV-2 glycoproteins and act as inhibitors (Borkotoky & Banerjee, 2021). Quercetin, a ubiquitous plant flavonoid, inhibits 3C-L protease and the M^{pro} as well as downregulates the ACE2 receptors (Derosa et al., 2021). Furthermore, some alkaloids such as 10-hydroxyusambarensine, cryptoquindoline, 6-oxoisoiguesterin, 22-hydroxyhopan-3-one, cryptospirolepine and isoiguesterin and terpenoids (6-Oxoisoiguesterin and 22-Hydroxyhopan-3-one) were identified as potential inhibitors of 3C-L protease (Gyebi et al., 2021).

The main challenge of phytopharmaceutical development for therapeutic claims is quality control, identification, and standardization of the bioactive compounds of plant-based products. In Uganda, Covidex and Covilyce 1 are plant–based products for the management of the symptoms of COVID-19. In addition, a similar product, Cugzin 400 mg (a combination of *Garcinia kola, Curcuma longa* and *Zingiber officinale*), has been officially listed in Nigeria for supportive care in COVID infection.

Therefore, this study aimed at identifying chemical constituents that possibly inhibit SARS-CoV-2 in a number of novel herbal preparations and ultimately develop standardized antiviral herbal-based products for the management of COVID-19 and related emerging as well as re-emerging viral threats. In a previous study, we have successfully reported a number of compounds, including natural products, with good inhibitory activity against the main protease enzyme (Loschwitz et al., 2021; Olubiyi et al., 2020). In the present work virtual screening against ten different macromolecular targets, and viral enzyme inhibition assay have been utilized in investigating the potential activities of eight herbal preparations against the SARS-CoV-2 virus.

2. Materials and method

2.1. Plant collection and preparation

2.1.1. Preparation of polyherbal VIVE, FORTE1 and Moringa seed extracts (MOK)

To investigate the claims on VIVE polyherbal dietary drink, which was available as a supportive care supplement during COVID-19, Rx Agroprocessing, Nigeria, kindly provided the product to researchers for scientific evaluation in June 2021. According to the product label, the plant component of the VIVE included; *Telfairia occidentalis* Hook. F (Curcubitaceae), *Sorghum bicolor* L. Moench (Poaceae), *Hibiscus sabdariffa* L. (Malvaceae), *Stigma maydis* L. (Poaceae) and *Beta vulgaris* L. (Amaranthaceae). Based on our recent extensive review on tropical antiviral plants (Attah et al., 2021), the following antiviral ethnomedicinal plants: *Andrographis paniculata* Nees. (Acanthaceae), *Terminalia catappa* L. (Combretaeae), *Bridelia ferruginea* (Euphorbiaceae) *and Scoparia dulcis* L. (Plantaginaceae), were selected based on evidence and used for the fortification of VIVE, transforming it into a novel polyherbal formulation known as FORTE1 for antiviral study. *Moringa oleifera* is a widely used Nigerian Traditional Medicine recipe especially during the pandemic. The seed kernel is chewed both as an antiviral prophylactic as well as for management of COVID-19; in addition to these antiviral claims, its inclusion in this study as a defatted and freezedried herbal preparation was based on claims of three COVID-19 positive Nigerians who took the preparation and tested negative after failing the viral screening for three consecutive times. The medicinal plants were obtained from Ilorin and Ibadan, Nigeria, identified and authenticated at the Forest Herbarium Ibadan (FHI), a unit of the Forestry Research Institute of Nigeria (FRIN). Voucher specimens with specimen numbers; FHI:113101, 113320, 113213, and 113217 respectively for *S. dulcis, M. oleifera, T. catappa,* and *A. paniculata* were deposited in the same

herbarium.

2.1.2. Preparation of COMBI-5

During the COVID-19 pandemic, our team explored "COMBI-5," an indigenous traditional herbal supplement, formulated and developed by Nestra Klinikal, Ibadan Nigeria. The herbal constituents which were used to formulate COMBI-5 include *Zingiber officinale* (Ginger), *Curcuma longa* (Tumeric), *Piper guineense* (black pepper), *Allium sativum* (Garlic) and *Xylopia aethiopica* (Negro pepper). This powdered blend of five common African spices had intriguingly showed promising results in a previous study when administered to patients who had COVID-19 (Onyeaghala et al., 2021, 2022). This study was aimed to scientifically evaluate its potential while respecting the cultural significance and intellectual property behind its creation. This exploration highlights the ongoing search for diverse healthcare solutions which are rooted in both tradition and scientific rigor.

2.2. Extraction of VIVE, FORTE1 and COMBI-5

VIVE is a polyherbal liquid formulation containing five medicinal plants and honey. This liquid formulation (200 mL) was freeze-dried (FD-12-MTP Floor Type Freeze Dryer, China) and refrigerated at minus 20℃ for bioassays. VIVE was formulated from *Beta vulgaris, Hibiscus sabdariffa, Sorghum bicolor, Stigma maydis*, and *Telfairia occidentalis* extracts (Table 1). In fortifying VIVE into FORTE1, *Andrographis paniculata, Bridelia ferruginea*, and *Terminalia catappa* were added. For COMBI-5, there were five plant constituents: *Allium sativum, Curcuma longa, Piper guineese, Xylopia aethiopica*, and *Zingiber officinale*. To extract COMBI-5 and the additional four plants (*S. dulcis* - SD, *B. ferruginea* - BRIDE, *T. catappa* - TC, *and A. paniculata* - AP) used to fortify and transform VIVE into FORTE1, finely powdered polyherbal COMBI-5 as well as the four FORTE1 plants (SD, BRIDE, TC and AP) were each extracted in 70 % ethanol, concentrated in vacuo (RE-2S-VD Rotary Evaporator, China) and Freeze-dried extracts were mixed in equal ratio to form the fortified mixture of antiviral preparation known as the "FORTE1". This was refrigerated until required for bioassay. The *Moringa oleifera* seed kernel (MOK) extract was obtained from 70 % ethanol following hexane defatting of the seed powder. Briefly, ~1 kg of finely powered seed kernel previously hulled was defatted using hexane. The dried marc was further extracted in ethanol containing 30 % of distilled water. The resulting filtrate (extract) was concentrated in vacuo using a Rotary evaporator and lastly freeze-dried and kept refrigerated at −20℃ until required for use. COMBI-5 is a powdered polyherbal combination containing five plant constituents commonly found in an average African kitchen as well as Traditional African Herbal homes/centers. Extract of COMBI-5 was obtained using 70 % ethanol in distilled water. The resulting filtrate was concentrated using the rotary evaporator (RE-2S-VD Rotary Evaporator, China), and freeze-dried with a laboratory lyophilizer. Freeze dried extract was kept refrigerated (−20 ℃) for biological assays.

Table 1
Plant and molecular composition of investigated plant extracts.

SN	Extract	Plants
1	VIVE	• *Beta vulgaris* • *Hibiscus sabdariffa* • *Sorghum bicolor* • *Stigma maydis* • *Telfairia occidentalis*
2	FORTE1	• *Beta vulgaris* • *Hibiscus sabdariffa* • *Sorghum bicolor* • *Stigma maydis* • *Telfairia occidentalis* • *Andrographis paniculata* • *Bridelia ferruginea* • *Terminalia catappa* • *Scoparia dulcis*
3	COMBI-5	• *Allium sativum* • *Curcuma longa* • *Piper guineese* • *Xylopia aethiopica* • *Zingiber officinale*
4	MOK	• *Moringa oleifera* kernel

2.3. Computational modeling

2.3.1. Ligand screening libraries

From an extensive search of multiple online research data repositories (Google Scholars, Pubmed, Google), we compiled a list of phytochemicals that have been reported in the plants from which VIVE, FORTE1, COMBI-5 and MOK were formulated. Finally, *Moringa oleifera* seeds were additionally investigated as the only constituent of MOK. Three dimensional structural models were subsequently generated for each phytoconstituent after which Gasteiger atomic charges were computed using the Autodock Tools (Derosa et al., 2021); the models were then saved as pdbqt files. For the VIVE series 3D models were generated for a total of 121 phytochemicals, while in the case of COMBI-5 the number was 139.

2.3.2. Macromolecular target library

To study how the plant chemical constituents would interact with SARS-CoV-2 at the molecular level, we retrieved the X-ray crystallographic structures for ten important SARS-CoV-2 macromolecular targets (See Table 2) starting with $3CL^{pro}$ the virus' main protease enzyme solved at 2.16 Å (6LU7.pdb) (Gyebi et al., 2021), and our main target of interest. We additionally retrieved for the SARS-CoV-2 the crystallographic structure for the S2 unit of spike protein solved at 2.90 Å (post fusion core, 6LXT.pdb) (Mann et al., 2020); RNA-dependent RNA

Table 2
Tabular description of the macromolecular targets employed for virtual screening.

SN	PDB Code	Target	Role in COVID-19 infection	Code (this article)
1	6LU7.pdb	main protease enzyme (nsp5)	A multifunctional protein with proteinase function that is responsible for transcription and replication of the SARS-CoV-2 virus.	$3CL^{pro}$
2	6LXT.pdb	Spike protein (S2 unit)	A post-fusion core structure that promotes the fusion of the virion and host cell membranes for subsequent host cell entry.	FusC
3	6M71.pdb	RNA-dependent RNA polymerase (nsp12)	Essential for viral replication and transcription.	Rdrp
4	7NNG.pdb	Helicase (nsp13)	Catalyzes NTP-dependent 5′−3′ direction nucleotide unwinding.	Heli
5	7DIY.pdb	Exoribonuclease (exoN, nsp14)	Mediate proofreading during replication.	ExoN
6	6NY1.pdb	Methyltransferase complex (nsp10/nsp16)	The enzyme complex is involved in viral mRNA capping as well as evasion of the host defense system.	PolP
7	6F06.pdb	Human cathepsin L	Plays an important role in viral entry via spike protein cleavage.	CatL
8	7MEQ.pdb	Human TMPRSS2	Essential for viral entry into host cells.	Tmp2
9	6WXC.pdb	Uridylate-specific endoribonuclease (nsp15)	Involved in the evasion of detection by the host defense system.	Endo
10	7JRN.pdb	Papain-like protease (nsp3)	Essential roles in viral replication.	Plpro

polymerase solved at 2.90 Å (6M71.pdb) while helicase was solved at 2.38 Å (7NNG.pdb) (Qi et al., 2021); nsp14-exoribonuclease domain solved at 2.68 Å (7DIY.pdb) (Sun et al., 2018); nsp10-nsp16 methyltransferase complex solved at 2.40 Å (6NY1.pdb) (Tahir ul Qamar et al., 2020); human cathepsin L solved at 2.02 Å (6F06.pdb) (Tarique et al., 2021); human TMPRSS2 solved at 1.95 Å (7MEQ.pdb) (WHO, 2021); and nsp15 uridylate-specific endoribonuclease solved at 1.85 Å (6WXC.pdb) (WHO, 2020); and papain-like protease (PLPro) solved at 2.48 Å (7JRN.pdb) (Wu et al., 2021). After downloading all target files from the www.rcsb.org, the files were prepared for use in virtual screening involving removal of atomic coordinates of extraneous co-crystallized units including crystallographic water molecules. Only the coordinates for the co-crystallized ligands (or substrates) and for the protein molecules were retained.

2.3.3. Virtual screening

In all ten macromolecular systems we employed the coordinates of the respective co-crystallized inhibitors/substrates on the macromolecular structures for automatically computing the docking grids using in-house Python script (Clement et al., 2021). The script employs the Cartesian coordinates of the co-crystallized inhibitor or substrate to locate the binding location around which the rectangular parallelepiped docking grid, also automatically computed by the script, is centered. In addition, grid volume is scalable by adjusting the separation d Å between the edge of the grid and the nearest atom of the co-crystallized inhibitor. By varying d between 1.5 Å, 3.0 Å and 5.0 Å three differently sized docking grids were generated per docking per macromolecular target for small, medium and big grids, respectively. As for the plants' natural products, Gasteiger charges were calculated for each the protein targets (for the reference bound inhibitors/substrates also) and the resulting coordinates saved in the PDBQT format. Using AutoDock Vina (Fernandez et al., 2021), each phytoconstituent was docked against each of the ten COVID19 macromolecules with the positional coordinates and bonded degrees of freedom of the receptors' binding site amino acids frozen in space while the torsions present in each docked plant constituent was optimized on the fly to obtain ligand conformation best fitting the receptor binding sites. The computation was performed for the three docking grid dimensions after which the grid with the best docking scores (best energy scores) across the docking spectrum was chosen for our analysis. The best binding free energies subsequently obtained for the ligand libraries were collected for analysis. Virtual screening was conducted for seven natural products library representing the different investigated tropical herbal materials including VIVE, FORTE1, COMBI-5, MOK, *Andrographis paniculata, Scoparia dulcis, and Terminalia catappa*.

2.4. Expression and purification of SARS-CoV-2 3CL^{pro}

SARS-CoV-2 3CL^{pro} (Uniprot entry: P0DTD1, virus strain: hCoV-19/Wuhan/WIV04/2019) was cloned, expressed, and purified as described previously (Eberle et al., 2021).

2.5. Activity assay and inhibition of SARS-CoV-2 3CL^{pro}

SARS-CoV-2 3CL^{pro} activity assay was performed using a fluorogenic substrate DABCYL-KTSAVLQ↓SGFRKME-EDANS (Bachem, Switzerland) in a buffer containing 20 mM Tris pH 7.2, 200 mM NaCl, 1 mM EDTA and 1 mM TCEP. The reaction mixture was pipetted in a Corning 96-Well plate (Sigma Aldrich) consisting of 0.5 μM protein and the assay was initiated with the addition of the substrate at a final concentration of 50 μM. The fluorescence intensities were measured at 60 s intervals over 30 min using an Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). The temperature was set to 37 °C. The excitation and emission wavelengths were 360 nm and 460 nm, respectively. All experiments were performed as triplicate and data are presented as mean ± SD.

Inhibition of SARS-CoV-2 3CL^{pro} activity by the plant extracts: TC (*Terminalia catappa*), BRIDE (*Bridelia ferruginea*), AP (*Andrographis paniculata*), SD (*Scoparia dulcis*), VIVE, FORTE1, COMBI-5 and MOK (*Moringa oleifera* kernel) was investigated using the activity assay described above. A primary inhibition test was performed using a plant extract with a final concentration of 50 μg/ml. A positive control was included to measure the maximum activity of the protease in the absence of the plant extracts. Moreover, inhibition controls were included by using 50 μM Curcumin (Merck, Germany) and 2.5 μM $ZnCl_2$ (Merck, Germany). For the final inhibition assays 0.5 μM of the protein was incubated with 0–10 mg/ml TC and BRIDE. 0–800 μg/ml AP and SD. 0–500 μg/ml FORTE1 and VIVE. 0–100 μg/ml COMBI-5. 0–40 μg/ml MOK. The mixtures were incubated for 30 min at RT and the assay was performed as described above. The IC_{50} value was calculated by plotting the initial velocity against various concentrations of the combined molecules using a dose-response curve in GraphPad Prism5 software. All measurements were performed with freshly purified protease and in triplicate, and data are presented as mean ± SD.

2.6. Statistical analysis

All experiments underwent at least 3 independent repetitions, and all data are expressed as the mean ± the standard deviations (SDs). The statistical significance of the mean values' differences was assessed with one-way analyses of variance (ANOVA), followed by Tukey's multiple comparison test. Significant differences were considered at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). All statistical analyses were performed with GraphPad Prism software version 5 (San Diego, CA, USA).

3. Results and discussion

Following the literature reports on the plants making up the studied novel herbal mixture, the phytochemical constituents of each herbal formulation was profiled for *in silico* antiviral screening. The studied herbal preparations include VIVE, FORTE1 (containing VIVE plus *Scoparia dulcis* L. Plantaginaceae, *Andrographis paniculata* Nees. Acanthaceae, *Bridelia ferruginea* Benth. Euphorbiaceae *and Terminalia catappa* L. Combretaceae), MOK and COMBI-5**.** Freeze-dried extracts of these herbal formulations demonstrated in vitro antiviral activities with varying potencies.

3.1. Molecular modeling

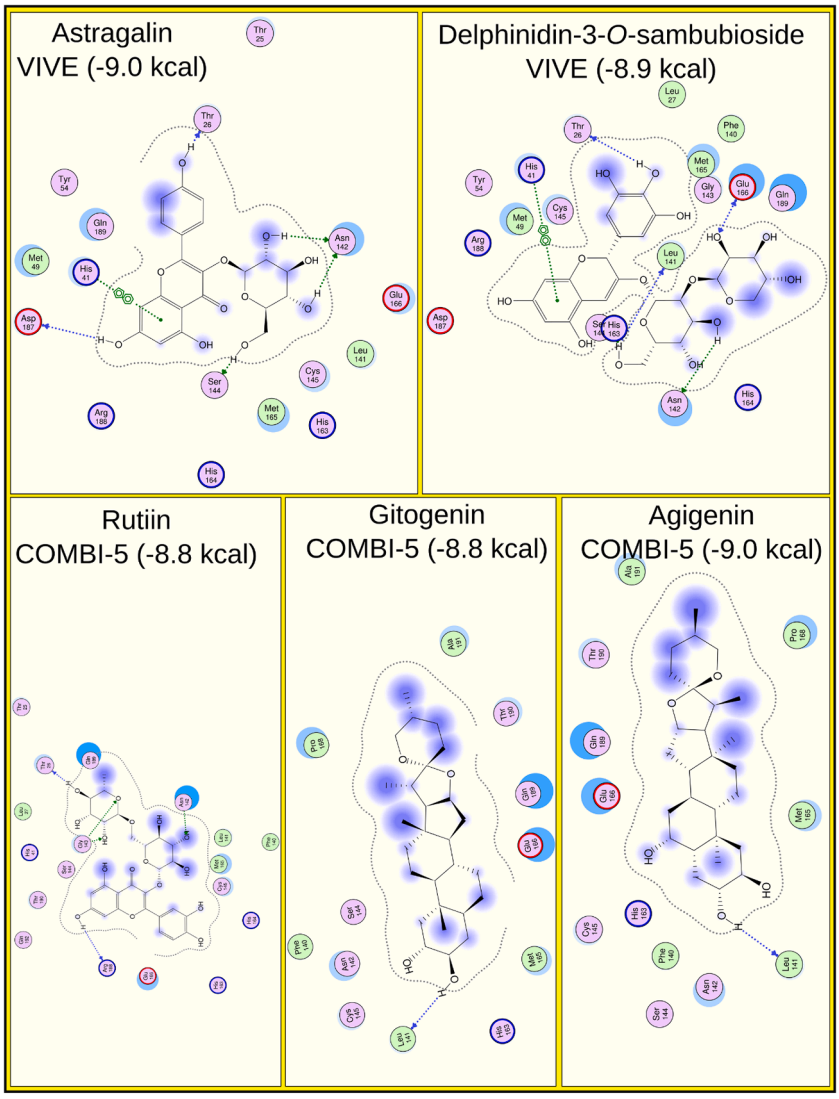
3.1.1. Natural products library virtual screening

A total of eighty-nine (89) natural products were screened in the VIVE library against ten SARS-CoV-2 macromolecular targets (Supplementary data Table S1). For 3CL^{pro}, astragalin and delphinidin-3-O-sambubioside with computed affinities of −9.0 and −8.9 kcal/mol, respectively were found to present the strongest binding interaction from the VIVE product. Saluretin (−8.8 kcal/mol) and miraxanthin V (−8.1 kcal/mol) were the highest binders for SARS-CoV-2 Plpro enzyme. The top two strong binders against FusC, PolP, Rdrp are beta-carotene (−8.8 kcal/mol) and Zeaxanthin (−8.5 kcal/mol), maysin (−9.4 kcal/mol) and gallotannins (−9.0 kcal/mol), Maysin (−8.9 kcal/mol) and 2-O-a-L-rhamnosyl-6-C-quinovosylluteolin (−8.8 kcal/mol), respectively. Strong binding (arbitrarily indicated by binding free energies lower than or equal to −8.0 kcal/mol) was generally not observed for EnDN, CatL, Helicase, ExonN and TMP2: observed binding strength for these five targets varied from mild to poor.

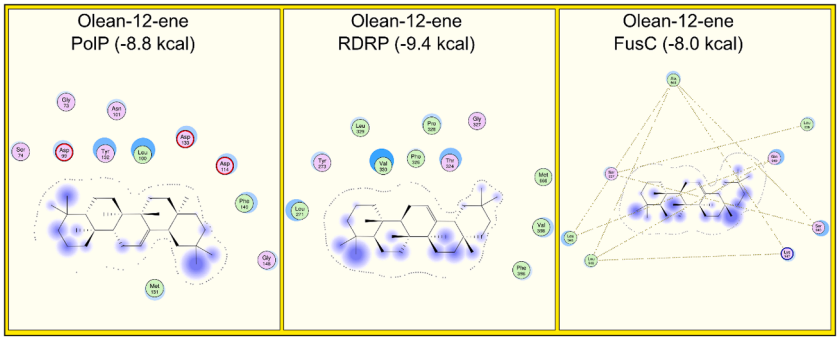
The fortification of VIVE brought the number of natural products in the virtual screening library of Forte1 to one hundred and twenty (120). In terms of computed strength of binding, the fortification does not produce any significant improvement in the number of ligands binding strongly to either of EnDN, CatL, Helicase, ExonN and TMP2; for which no strong binders were identified in the Vive screening (Table S2). While the top binders remained the same for 3CL^{pro} [astragalin (−9.0 kcal) and

delphinidin-3-O-sambubioside (−8.9 kcal)], FusC [B-Carotene (−8.8 kcal) and Zeaxanthin (−8.5 kcal)], PolP [maysin (−9.4 kcal) and gallotannins (−9.0 kcal)], in the case of Plpro and Rdrp, an improvement in the number of strong binders relative to the VIVE profile was observed. Ellagic acid with computer −8.6 kcal affinity was found in addition to saluretin (−8.8 kcal/mol) and miraxanthin V (−8.1 kcal/mol) in the screening against Plpro, while in the case of Rdrp, chebulagic acid and isocorilagin, both with computed affinities of −9.0 kcal, were identified alongside the original top performers [Maysin (−8.9 kcal) and 2-O-a-L-rhamnosyl-6-C-quinovosylluteolin (−8.8 kcal)]. In principle, this should indicate a gain of Plpro- and Rdrp-targeted antiviral principle in FORTE1 compared to VIVE.

The COMBI-5 ligand library employed contains 138 members, which are screened against the panel of SARS-CoV-2 pharmacological targets



A



B

Fig. 1. **A**: Interaction of VIVE's Astragalin and delphinidin-3-*O*-sambubioside and COMBI-5's rutin, gitogenin and agigenin with binding site residues of $3CL^{pro}$. **B:** Interaction of MOK's Olean-12-ene with amino acid residues of the PolP (methyltransferase), RDRP and fusion core protein (S2 unit of the spike protein).

(Table S3). The interaction profile computed for COMBI-5 presents an overall interesting picture with much fewer positive binding free energy values (i.e. less unfavorable binding) compared with the VIVE formulations. On the average therefore, and deriving directly from the greater number of favourable binders found in COMBI-5, it will be expected to exhibit stronger antiviral effect against the virus. Interestingly, as observed in the VIVE product series, significant binding affinities (<= −8.0 kcal) were also not observed against EnDN, CatL, Helicase, ExonN and TMP2. The top strong binders reported against $3CL^{pro}$ are apigenin (−9.0 kcal), gitogenin/rutin (−8.8 kcal), and for Plpro 4, 5, 6, 6alpha-Tetradehydro-7-oxoaporphine (−9.1 kcal) and liriodenine (−9.0 kcal) were obtained. Agigenin (−8.1 kcal) and tigogenin (−8.0 kcal) were found for FusC, 1,2,3,10-Tetramethoxy-9–2-hydroxy-4.5-dimethoxybenzyloxyoxoaporphin (−8.7 kcal) and tigogenin (−8.3 kcal) for PolP, while Rdrp had tigogenin (−8.8 kcal) and gitogenin (−8.5 kcal) as the best two strong binders. In comparing these with the profiles obtained for VIVE product series reveals at par overall interaction strength (using the strongest binders) in their interaction with $3CL^{pro}$, while the VIVE products show marginal stronger interaction with FusC, PolP and Rdrp. COMBI-5 on the other hand was better in associating with Plpro.

A similar interaction pattern of preferential binding to $3Cl^{pro}$, Pl^{pro}, FusC, PolP and Rdrp was to different degrees recorded for the MOK (Table S4), *Scoparia dulcis* (Table S5), *Terminalia catappa* (Table S6), and *Andrographis paniculata* (Table S7) natural product libraries. In the case of MOK, it is significant to notice that two natural products were for the first time found that strongly inhibited ExoN with binding free energy less than and equal to −8.0 kcal (Table S4). These are Olean-12-ene with computed affinity of −8.1 kcal, and campesterol with affinity of −8.0 kcal. In fact, all strong binding interactions observed in MOK involved Olean-12-ene that was found to bind FusC, PolP and Rdrp with −8.0, −8.8, and −9.4 kcal affinities, respectively. In the case of Rdrp, the highest binding strength reported so far in this work is for Olean-12-ene, which is superior to any natural products of VIVE products and COMBI-5.

3.1.2. Binding site interactions

Astragalin and delphinidin-3-*O*-sambubioside of VIVE and rutin, gitogenin and apigenin of COMBI-5 formed an overall pattern of binding site interaction with the main protease enzyme involving mainly hydrophobic contacts, hydrogen bonding and pi-interactions (Fig. 1A). Numerically the highest number of contacts, 18, 17 and 16 amino acids, was observed with rutin, delphinidin-3-*O*-sambubioside, and astragalin, respectively: all three of which are glycosylated flavonoids. The number of binding site contacts appears to be positively correlated with the number of sugar units with all three possessing two sugar units, except astragalin that has only one sugar unit. Binding affinity on the other hand is marginally correlated with the number of sugar units with astragalin presenting the strongest binding interaction out of the three. However, with the binding free energy difference being no greater than ± 0.2 kcal, it might be correctly adjudged as statistically insignificant. The two steroidal sapogenins, gitogenin and apigenin, even though smaller in molecular mass relative to the flavonoids, did not perform more poorly. In fact, the computed binding free energy values are comparable to the values obtained for the flavonoids. SARS-CoV-2 main protease enzyme features a very large binding site (Olubiyi et al., 2020), for which the smaller sapogenins can be said to possess qualitatively higher binding efficiencies than the glycosylated flavonoids. It is expected that this could have contributed in no small measure to the overall better inhibitory profiles obtained in this work for COMBI-5 compared with VIVE. Independent investigation by Olubiyi et al. (Olubiyi et al., 2020, 2022) and Loschitwz et al. (Loschitwz et al., 2021) indicated that, beyond an advantageous computed affinity, good main protease inhibitors should form specific binding interaction with the His41-Cys145 catalytic dyad of the viral enzyme. While all five natural products interacted via hydrophobic contact with Cys145, only astragalin, delphinidin-3-*O*-sambubioside (both of VIVE) and rutin form specific contact with His41 involving both pi- and hydrophobic interactions (only the later in the case of rutin). These three metabolites could be further investigated for their inhibitory potentials in purified form.

A total of eleven Pl^{pro} binding site amino acids were involved in the binding of the top performing products from VIVE and COMBI-5 ligand libraries (Figure S1). Of this hydrogen bonding interaction was observed in saluretin contact with Asp164 and Tyr268; miraxanthin V contact with Asp164, Gln269, Tyr273 and Thr301; and liriodenine contact with Tyr273. 4,5,6,6a-

Tetradehydro-7-oxoaporphine formed exclusively hydrophobic contacts. VIVE library products represented by saluretin and miraxanthin V involve small few ring and flexible systems. Their higher polarity allows for a higher degree of hydrogen bonding contacts compared with the COMBI-5 library. The sulphonamide moiety of saluretin forms two hydrogen bonds with Tyr268 while the lactam forms another hydrogen bond with Asp164. Three hydrogen bonding contacts were shared between miraxanthin V's two catechol OH groups and Asp164, Thr301 and Tyr273. In the case of 4,5,6,6a-Tetradehydro-7-oxoaporphine and liriodenine, their large fused ring systems coupled with the essential absence of polar groups make polar contacts of much less importance compared with hydrophobic interactions. The exemption is the two hydrogen bonding contacts of liriodenine with Tyr273 and Thr301, and even then, liriodenine is marginally less efficient at binding the receptor compared with the more hydrophobic 4,5,6,6a-Tetradehydro-7-oxoaporphine as indicated by the affinity data.

The interaction of the representative natural products with the activity site of methyltransferase complex (nsp10/nsp16) suggests a preference for polar functional groups as seen in maysin, gallotannin, and 1.2.3.10-Tetramethoxy-9–2-hydroxy-4.5-dimethoxybenzyloxyoxoaporphine (Figure S2). While it might be pointed out that disproportionately high hydrophilicity might severely hamper bioavailability at the enzyme active site under in vivo setting, this analysis is useful for sounding the chemistry of potential inhibitors. Also, for polyherbal mixtures as investigated in this study, attainment of high local concentration for the individual constituent natural products is not as critical as when single compounds are administered. All four ligands engage polar interactions with the binding site amino acids most of which are hydrophilic polar residues, and nearly all ligands engaged Asp130 in particular hydrogen bond interaction.

For RDRP two doubly glycosylated flavonoids differing only in their sugar units, maysin and 2-*O*-a-L-rhamnosyl-6-C-quinovosylluteolin, topped the performance list for VIVE while two sapogenins, tigogenin and gitogenin, dominated COMBI-5′s performance profile (Figure S3). The more polar glycosylated flavonoids of VIVE again preferably enlist hydrogen bond formation in addition to hydrophobic contacts while the tigogenin and gitogenin exclusively relied on the later in forming strong interaction with the enzyme's binding site. In their interaction with the fusion core protein of the S2 unit of the spike protein of SARS-CoV-2, a slightly different pattern was observed in the top performing products of VIVE, involving top-performing hydrophobic products-beta-carotene and zeaxanthin (Fig. 1B). Tigogenin and gitogenin were again the best performing constituents of COMBI-5.

In summary, the best binding affinities in the different polyherbal libraries were recorded for SARS-CoV-2′s papain-like protease, main protease, the fusion core unit of the spike protein (S2 unit), methyltransferase (PolP), and the RNA-dependent RNA polymerase (Rdrp). This strongly suggests multiple roles for the products in viral replication and transcription, viral entry as well as in rendering host immunity cooperative for the infection process. MOK additionally possesses metabolites that are capable of inhibiting some of the other targets; but deficiencies in the physicochemistry (e.g. Olean-12-ene) may present a barrier to their access, in vivo, to the pharmacological targets.

3.2. Screening of extracts of medicinal herbs for inhibition of SARS-CoV-2 3CL^{pro} activity

Medicinal herbs' extracts form a reservoir for bioactive compounds and present a strong benefit to human health (Proestos, 2020). Besides, plant extracts polyphenolic compounds show inhibition of the SARS-CoV-2 3CL^{pro} activity, e.g. Quercetin (Derosa et al., 2021), epigallocatechin gallate, ellagic acid, rhoifolin (Loschwitz et al., 2021), curcumin and resveratrol (Bahun et al., 2021). The residual activity of SARS-CoV-2 3CL^{pro} in the presence of eight medicinal herb extracts (TC, BRIDE, AP, SD, VIVE, FORTE1, COMBI-5 and MOK) was tested at a final concentration of 50 μg/ml. Two of the extracts showed low inhibitory potential and led to average residual protease activities around 80 % (TC and BRIDE). AP and SD displayed intermediate inhibitory capacity (30–50 %). Finally, four extracts were found to show high inhibitory potential against SARS-CoV-2 3CL^{pro} and reduced the activity about 70 % (VIVE and FORTE1), ~ 95 % COMBI-5. MOK showing a complete inhibitory capacity, 0 % of 3CL^{pro} residual activity at the concentration of 50 μg/ml (Fig. 2). Additionally, two inhibitor controls were performed; curcumin and $ZnCl_2$ possess a proven inhibitory effect against the SARS-CoV-2 3CL^{pro} activity (Bahun et al., 2021; Grifagni et al., 2021). Curcumin inhibited the protease activity at the tested concentration > 95 % and $ZnCl_2$ > 98 %. The inhibitory effect of MOK and COMBI-5 show a similar strength against the protease activity as Curcumin and $ZnCl_2$.

Ryu and colleagues showed that *Tripteryguim regelii* extracts inhibited SARS-CoV 3CL^{pro} activity >70 % at 30 μg/ml (Ryu et al., 2010), which is in a similar range to the SARS-CoV-2 3CL^{pro} inhibition effect for VIVE, FORTE1, COMBI-5 and MOK.

Additional experiments (Fig. 3i, ii, iii; Figure S1-S9) of the inhibitory effects of the eight medicinal plant extracts were carried out, therefore 0–10 mg/ml TC and BRIDE; 0–800 μg/ml AP and SD; 0–500 μg/ml FORTE1 and VIVE; 0–100 μg/ml COMBI-5 and 0–40 μg/ml MOK were tested against the SARS-CoV-2 3CL^{pro} activity. MOK inhibited 100 % of SARS-CoV-2 3CL^{pro} activity at a concentration of 40 μg/ml and COMBI-5 at 100 μg/ml (Figs. 2 and 3iii). FORTE1, VIVE, AP, SD, BRIDE and TC inhibited the protease activity at concentrations higher than 450 μg/ml. The calculated IC_{50} for the medicinal herbs' extracts are shown in Table 3 (The corresponding dose response curves for the IC_{50} determination are shown in Fig. 3i, ii, iii; Figure S1-S9); MOK and COMBI-5 possess IC_{50} values < 4 μg/ml.

The determined IC_{50} values of the eight medicinal herbs' extracts are in similar concentration ranges to already published plant extracts which inhibited the SARS-CoV-2 3CL^{pro} activity, turmeric extract (IC_{50}: 15.74 μg/ml), mustard seed extract (IC_{50}:128.1 μg/ml) and wall rocket extract (IC_{50}:257.4 μg/ml) (Guijarro-Real et al., 2021). The individual plant components of novel herbal preparations reported in this study are among ethnomedicinal plants commonly used in Nigeria (Abubakar et al., 2022) whose antiviral activity have also been documented. Many of these studies used cell lines to investigate the antiviral activities of extracts of these antiviral plants (Perera et al., 2021; Xiong et al., 2022, 2021; Kathum et al., 2024). Mechanistic explanation has been provided for chemical constituents of some of the plants such as for sulphur compounds in *Allium sativum* (Jikah & Edo, 2023). Despite defatting the Moringa seed used in this study to exclude reported toxicity (Attah et al., 2022), the resulting extract remained potent indicating the presence of active metabolites within extract used. Xiong and colleagues (Xiong et al., 2022, 2021) reported Moringa A and two new antiviral compounds in Moringa seed. While terpenes have been documented to possess potent antiviral property (Darshani et al., 2022), specific analogues of Olean-12-ene have been reported as potent inhibitors of envelop viruses (Shao et al., 2023) as observed in this study. Giugliano et al. (2024) recently reported the potential of Moringa in combating respiratory diseases.

Findings from our in vitro experiment showed that the most active herbal preparations used in this study, VIVE (17.3 ± 1.4 μg/ml), COMBI-5 (IC_{50} - 0.9 ± 0.1) and MOK (IC_{50} −3.6 ± 0.9) are rich in antiviral specialized metabolites such as beta-carotene, Zeaxanthin, maysin, gallotannins, luteolin, astragalin, delphinidin, gallotannin, Ellagic acid, chebulagic acid and corilagin, Olean-12-ene, campesterol, rutin, gitogenin and apigenin. *In silico* target analysis of all the documented antiviral metabolites against ten pharmacological targets on the SARS COV-2 prioritized these compounds from a list of over 370 specialized metabolites for their potent inhibition of five out of the ten SARS COV-2 viral enzymes screened.

These macromolecular viral targets which the metabolites inhibited *in silico* include 3CL^{pro}, FusC, Rdrp, PolP and Plpro. This means the identified metabolites in VIVE, COMBI-5 and MOK may selectively be acting via interaction with and inhibition of these ezymes. The mechanisms being the respective inhibition of transcription and replication of

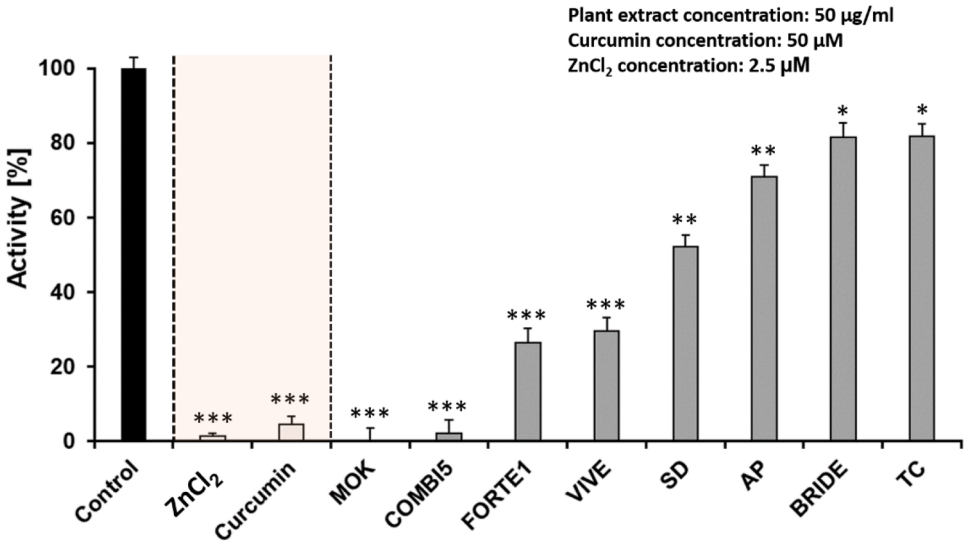


Fig. 2. Screening for the residual activity of SARS-CoV-2 3CL^{pro} in the presence of eight extracts of medicinal herb at a concentration of 50 μg/ml. The inhibitor controls curcumin at a concentration of 50 μM and $ZnCl_2$ at 2.5 μM inhibit the protease activity >95 %. Polyherbal COMBI-5 inhibit the virus protease activity >95 % and MOK (defatted Moringa seed kernel extract) inhibit the protease activity 100 %. *S. dulcis* - SD, *B. ferruginea* - BRIDE, *T. catappa* - TC, *and A. paniculata* – AP. Data shown are the mean ± SD from 3 independent measurements ($n = 3$). Asterisks mean that the data differs from the control (0 μM inhibitor) significantly at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), level according to ANOVA and Tukey's test.

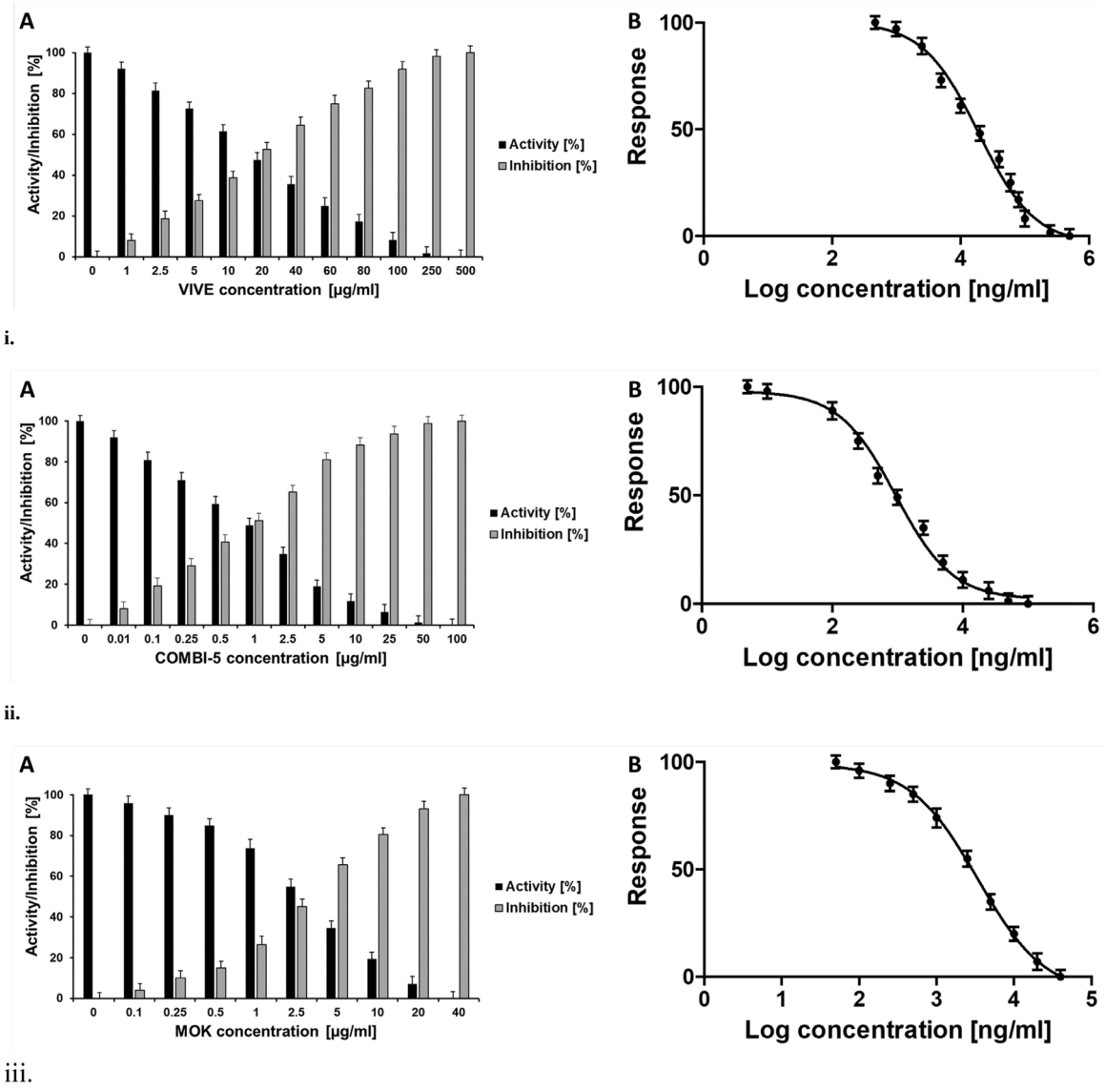


Fig. 3. i. Inhibition effect and dose response curve of VIVE polyherbal over SARS-CoV-2 $3CL^{pro}$. A: Normalized activity and inhibition of SARS-CoV-2 $3CL^{pro}$ under VIVE influence. B: Dose response curve of Vive and SARS-CoV-2 $3CL^{pro}$. The normalized response [%] of SARS-CoV-2 $3CL^{pro}$ is plotted against the Log of the VIVE concentration.

ii. Inhibition effect and dose response curve of COMBI-5 polyherbal over SARS-CoV-2 $3CL^{pro}$. A: Normalized activity and inhibition of SARS-CoV-2 $3CL^{pro}$ under COMBI-5 influence. B: Dose response curve of COMBI-5 and SARS-CoV-2 $3CL^{pro}$. The normalized response [%] of SARS-CoV-2 $3CL^{pro}$ is plotted against the Log of the COMBI-5 concentration.

iii. Inhibition effect and dose response curve of MOK (defatted Moringa seed kernel extract) over SARS-CoV-2 $3CL^{pro}$. **A:** Normalized activity and inhibition of SARS-CoV-2 $3CL^{pro}$ under MOK influence. **B:** Dose response curve of MOK and SARS-CoV-2 $3CL^{pro}$. The normalized response [%] of SARS-CoV-2 $3CL^{pro}$ is plotted against the Log of the MOK concentration.

Data shown are the mean ± SD from 3 independent measurements ($n = 3$).

Table 3
Summary of the SARS-CoV-2 $3CL^{pro}$ inhibition experiments by tested extracts of medicinal herbs.

Plant extract	IC_{50} ± STD [µg/ml]
TC	372.2 ± 28.6
BRIDE	494.9 ± 19.6
AP	81.7 ± 3.2
SD	53.5 ± 2.9
VIVE	17.3 ± 1.4
FORTE1	21.5 ± 1.1
COMBI-5	0.9 ± 0.1
MOK	3.6 ± 0.9

the SARS-CoV-2 virus ($3CL^{pro}$, Pl^{pro}, Rdrp), fusion of the virion and host cell membranes for subsequent host cell entry (FusC), viral mRNA capping as well as evasion of the host defense system (PolP). These multitarget mechanism-based activity of these tropical indigenous antiviral phytomedicines highlights their potential advantage over the single target-based synthetic drugs. The desirable antiviral activity demonstrated by VIVE, COMBI-5 and MOK validates their wider usage during the peak of the COVID-19 pandemic. For instance, the selection of MOK for antiviral studies was based on the claims of three Nigerian travelers who, due to a positive COVID-19 test, could not go for visa interviews after three consecutive screening. However, after taking 1 g (x3) of the freeze-dried MOK for two days, their PCR test came out negative. Interestingly, in this study, MOK produced the best and complete inhibition of the viral enzyme. This is a testament to the reliability

of traditional knowledge widely used in indigenous populations. However, the long-term safety of MOK (Attah et al., 2022) as well as the other two potent novel herbal preparations must be well validated in human populations for a possible future clinical antiviral application. Moreso, individual active metabolites reported in this study such astragalin, delphinidine, rutin, olean-12-ene, campesterol deserve further in-depth scientific investigation for a potential antiviral drug development.

4. Conclusion

While the use of traditional herbal remedies in Nigeria during the COVID-19 pandemic and beyond is well-documented, there is a need for more rigorous scientific investigation to fully understand their antiviral potential and mechanisms of action (Attah et al., 2021; Oladele et al., 2020). In this work, we have demonstrated that VIVE, COMBI-5 and MOK have scientific basis for their use during and after the COVID-19 pandemic. The antiviral activities of these dietary herbal preparations appear to be associated with metabolites such as flavonoids and terpenes reported in the plants. Elaborate phytochemical analysis and mechanism-based standardization of these antiviral herbal preparations as well as the implicated specialized metabolites will provide reliable scientific evidence and thus support their global acceptance and application in clinical settings.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

All data will be made available upon reasonable request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2025.100969.

Data availability

All data not available in the supplementary file will be provided upon request.

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