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Original Research Article

Immunomodulatory Activity of the Aqueous Extract of the pith of *Citrus limon* L. (Rutaceae) Using Cyclophosphamide Induced Myelosuppression

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ABSTRACT

The genus Citrus species have been widely used in the relief of arthritic pain in traditional medicine. *Citrus limon* pith was one of the constituents of herbal decoctions taking daily for prevention against Coronavirus disease during covid -19 era. This study investigated the immunomodulatory activity of the aqueous extract of the pith of *C. limon* using cyclophosphamide-induced myelosuppression and delayed type hypersensitivity reaction in Wistar rats. Thirty-six rats were divided into six groups of six animals each. Three groups served as the control while the remaining three groups were given 150, 300, 600 mg/kg of extract respectively via oral route for 13 days. Groups II to VI were administered cyclophosphamide 30 mg/kg i.p on day 11, 12, and 13. On the 14th day, blood samples were collected for haematological parameters analysis. Delayed type hypersensitivity reaction was carried out using 24 animals divided into four groups of six animals each. Phytochemical screening revealed the presence of triterpenoids, flavonoids, and carbohydrates. LD_{50} was found to be greater than 3000 mg/kg. *C. limon* extract on cyclophosphamide-induced myelosuppression showed a statistically significant ($P<0.05$) increase in the white blood cells (WBC), platelets and lymphocytes counts at all extract doses. In the delayed type of hypersensitivity reaction, percentage increase in inflammation was 24.3%, 31.5% and 43% at extract doses of 150, 300 and 600 mg/kg. The findings of this study have shown that the aqueous extract of the pith of *C. limon* possessed immunostimulatory activity.

Keywords: *Citrus limon*, immunomodulatory, cyclophosphamide, delayed hypersensitivity, inflammation .

Introduction

Presently, the world is facing a problem of high population growth, increase in the incidence of diseases, unemployment and a shift in ethical values which has affected the quality of lives.[1] These, together with the attendant co-morbidity of immune disorders have recently stimulated research interest in immune boosting potentials of medicinal plants. [2]

Researchers are now interested in immunomodulation due to the emergence of antibiotic resistance by bacteria, and antiviral resistance by viruses and modulation of immune response to alleviate disease conditions is now being recognized as a key component of disease control. [3] Extracts of whole plant or plant parts have been and are still being widely investigated in different parts of the world for their potential immunomodulatory properties. [4]

Coronavirus disease caused by severe acute respiratory syndrome coronavirus- 2 was a pandemic from the year 2020 till early 2021 and. is characterized by fever and respiratory syndrome. It has affected almost 659 million people worldwide. [5]

The phytoconstituents of medicinal plants such as flavonoids, glycosides, terpenoids and alkaloids have been found to be accountable for the observed immunomodulatory activity of these plants. [6]

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The concerns about the numerous side effects of conventional drugs have stimulated a profound interest in the use of natural products as an alternative to conventional treatment in management and treatment of various diseases. [7]

It has been reported that herbal medicines could be considered an alternative approach for the treatment and prevention of COVID-19. [8,9] Recent systematic review articles also concluded that herbal medicine showed significant effects in increasing the total effective rate and alleviating the symptoms.[10-12]

Citrus limon pith was one of the medicinal plants combined with other plants as a decoction taken orally daily during the COVID-19 era as a preventive herbal preparation.

Citrus limon (lemon) is one of the species from the genus Citrus. It is an evergreen tree in the family of Rutaceae grown for its edible fruit [13] which among other things, are used in variety of foods, drinks, cosmetics, home-made remedies, and traditional medicine.[14]

Citrus limon has found a wide range of uses especially among the locals of Southern, Eastern and Western Nigeria. *Citrus limon* is commonly known as Oroma nkirisi (Igbo), Orombo-wewe (Yoruba), lemun tsami (Hausa).

Citrus species are commonly used as food ingredients due to its high nutritious value. *C. limon* juice has been used as a remedy for scurvy in traditional medicine and has been found useful in the control and treatment of high blood pressure, common cold, and irregular menstruation. The essential oil of *C. limon* is a known remedy for cough.[15] Several studies have shown that lemon juice, essential oils and extracts of lemon possess antioxidant, anti-inflammatory, antibacterial, anticancer, antihyperlipidemic and neuroprotective properties and have been attributed to the presence of citric acids, ascorbic acid, and other essential oils.[16,17] However, there is no scientific report on the immunomodulatory activity of the *C.limon* pith.

Thus, this study was aimed at investigating the immunomodulatory activity of aqueous extract of *C. limon* pith in Wistar rats.

Materials and Methods

Plant material
The fresh fruits of *Citrus limon* were purchased from Choba Market in Obio-Akpor Local Government Area of Rivers State and was identified at the herbarium of the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmaceutical Sciences, University of Port Harcourt by Dr. Suleiman Mikailu, where a voucher specimen was deposited (UPH/R/0456).

Preparation of extract
The fresh fruits of *C. limon* were cleaned thoroughly with water to clear it of dirt. The cleaned fruits were then peeled and exocarp removed, cut into sections removing the juice sacs, pulp and seeds exposing the white mesocarp which is known as pith. An aqueous decoction of *C. limon* pith was prepared using an extraction ratio of 1:4.[18]
Plant material of 75 g weight was blended with aliquots of de-ionized water from the measured 300ml using an electric blender and transferred into a stainless-steel pot. The remaining water was added, and it was boiled for about 10 minutes, filtered using a clean muslin cloth, and a clear filtrate was obtained. This procedure was done daily.

Animals used
A total of 66 Wistar rats of both sexes with average weight of 165g were obtained from the animal house of the Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt. The animals were housed at the animal house of the Department of Experimental Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Port Harcourt with free access to standard commercial diet and water ad libitum. The animals were fasted overnight prior to the experiment. This study was carried out in compliance with international ethics and guidelines on animal use and experiments. Ethical approval with reference number UPH/CEREMAD/REC/MM85/026 was obtained from the University of Port Harcourt research ethics committee.

Phytochemical Screening
Qualitative phytochemical analysis of the aqueous extract of *C. limon* pith was performed using test protocols outlined by Trease and Evans [19] to ascertain the phytochemical constituents.

Evaluation of acute toxicity
Acute toxicity determination was conducted as per OECD guideline 425 method to assess the median lethal dose (LD_{50}) of the aqueous extract of *C. limon* pith. This was carried out via oral route of administration.[20]
A total of six rats of both sexes were used. The animals were divided into two groups of three animals (3 males in one group and 3 females in the second group). The freshly prepared aqueous extract of *C. limon* pith was administered to the six animals at a dose of 3000 mg/kg body weight. The animals were closely monitored for signs of toxicity and death for a period of 14 days and observations were recorded.
The doses for the study were selected based on the outcome of the acute toxicity studies carried out. The animals did not show any mortality at the dose of 3000 mg/kg and hence, its 1/20th dose (150 mg), 1/10th dose (300 mg), 1/5thdose (600 mg) were used as therapeutic doses for the aqueous extract of the study.

Antigen preparation
Blood samples were obtained from the jugular vein of a healthy sheep maintained in the livestock farm of the Faculty of Agriculture, University of Port Harcourt, Rivers State, Nigeria, and put into a sodium citrate anticoagulant tube. The red blood cells were then washed thrice with copious volume of sterile normal saline by centrifugation at 3000 RPM for 15 minutes. The final cell volume was adjusted to a concentration of 1 x 10^9 cells/ml and used for immunization and challenge.

Cyclophosphamide-induced myelosuppression
A total of 36 Wistar rats of both sexes were used. Animals were divided into six groups of six animals each with males separated from the females. Group 1 served as the untreated group, received the vehicle (normal saline) for a period of 13 days. Group II served as the negative control group, were administered with normal saline for 13 days and each animal was injected with cyclophosphamide (30 mg/kg i.p) on the 11th, 12th, and 13th day. Group III served as the positive control, received levamisole (50 mg/kg), and on the 11th,12th, 13th day were injected with cyclophosphamide (30 mg/kg i.p). Group IV, V, VI were administered aqueous extract of the pith of C. limon at 150, 300, 600 mg/kg bodyweight via oral route respectively for 13 days. Group IV, V, VI were injected with cyclophosphamide (30 mg/kg i.p) on the 11th, 12th, 13th days, one hour after the administration of the extract. Blood samples were collected on the 14th day of the experiment through cardiac puncture for haematological parameters (red blood cells, platelets, white blood cells and differential leucocytes counts) analysis.[21]

Haematological parameters evaluation
The analysis of haematological parameters was performed using procedures outlined by Ochei and Kolhatkar.[22]

Packed Cell Volume (PCV)
The hematocrit tube was filled with anticoagulated blood using a stemmed Pasteur pipette. It was then centrifuged at 3000RPM for 30 minutes and read with hematocrit reader in percentage.
PCV (%) = (packed RBC column height/ total blood column height) x100

Hemoglobin (Hb) Cyanmethaemoglobin method
A 0.02 ml of blood was added to 5.0 ml of Drabkin's solution in a test tube, mixed well and allowed to stand for 10 minutes for full color development. Absorbance was read colorimetrically at 540nm against a blank Drabkin's solution.
Hb(g/dl) = (Absorbance of test/ Absorbance of standard) x concentration of standard.

Red blood cell Count
A 1:200 dilution of blood was prepared with diluting fluid (formal Citrate Solution) using a Thoma pipette (0.02 ml blood + 3.98 ml fluid). The improved Neubaur counting chamber was charged with mixed blood. Cells were allowed to settle in the chamber for at 5 minutes. The ruled area of the chamber was located using x40 objective lens under the microscope.
RBC/mm^3= number of cells counted x (1/area counted(mm^2) x (1/depth(mm)) x dilution factor.

White blood cell Count
A 1:20 dilution of blood was prepared with diluting fluid (2% acetic acid) using a Thoma pipette (0.02 ml of blood +0.38 ml of fluid). The Neubaur counting chamber was charged with the diluted blood and allowed to settle for 5 minutes. The ruled 4mm^2 squares were located using x10 objective of the microscope and the cells counted.
WBC/ mm^3= number of cells counted x (1/area counted(mm^2) x (1/depth(mm)) x dilution factor

Platelets count
A 1:200 dilution of whole blood was prepared with diluting fluid (formal citrate) using Thoma pipette (0.02 ml of blood +3.98ml of fluid). Platelets were allowed to settle in the moist chamber for 5 minutes. Using x10 objective of the microscope, the ruled squares were located. Platelets appear highly refractile particles. The number of platelets was counted using x40 objective in the four large corner squares 4mm^3.
Platelets count/mm^3 = number of platelets counted x (1/area counted(mm^2)) x (1/depth (mm)) x dilution factor.

Differential count of Leucocytes
A thin blood film, dried in air and fixed in methyl alcohol for 3 minutes. 1 in 10 volumes of Giemsa stain was prepared, used to flood the slide, and allow to stand for 15 minutes. It was then washed and differentiated with the buffer solution, dried in air, and examined microscopically. The different leucocytes were identified and counted using leucocyte

counter. The differential count of the leucocytes is expressed as the number of each type per 100 white cells.

Erythrocyte sedimentation rate
A Westergren ESR tube and stand was used. The whole blood was aspirated into the westergren tube to the top mark. The tube was placed vertically in a stop rack with spring clip and left for an hour, and the cell settlement was read against the graduation mark.

Evaluation of delayed type hypersensitivity (DTH) response
A total of 24 albino rats of either sex was allotted into four groups of six animals each. Group I served as the control group and received distilled water. Group II, III, IV received aqueous extract of *C. limon* pith at doses 150, 300, 600 mg/kg body weight respectively via oral route. On day zero, the rats were treated with the extract and sensitized by intradermal injection of 0.1 ml of 1x 10^9 cells/ml freshly prepared sheep red blood cells (SRBCs) into the right hind foot paw. Administration of extract continued daily for 7 days. On day 7, the diameter of the left hind paws of the rats were measured using a digital vernier caliper after which the rats were challenged by intradermal injection of 0.1 ml of 1 x 10^9cells/ml freshly prepared SRBCs into the left hind foot paw. The diameter of the left hind paw was measured again with a digital vernier caliper 24 hours after the challenge.[23] Delayed type hypersensitivity response was calculated as percentage increase in paw oedema. [24]
Percentage increase=
(Footpad thickness after antigen challenge-footpad thickness before antigen challenge/footpad thickness before antigen challenge) ×100

Statistical analysis
All the data obtained were expressed as mean ± SEM. Statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by LSD post hoc test using SPSS version 23. The statistical analysis was done to determine the significance between control group and treated groups. P-values were considered statistically significant at a value of P< 0.05.

Results and Discussion

Phytochemical Screening
Qualitative phytochemical analysis of the aqueous pith extract of *C. limon* revealed the presence of flavonoids, triterpenoids, reducing sugars and carbohydrates while anthraquinones, alkaloids, tannins, cardenolides, cyanogenic glycoside and saponins were absent. Flavonoids are polyphenolic with a wide range of structures[25] and are potent water-soluble super antioxidants and free radicals' scavengers which prevent oxidative cell damage and have strong anticancer activity.[26] Flavonoids have been shown to exhibit significant immunomodulatory and anti-inflammatory activities.[27] Triterpenoids have been observed to possess anticancer, anti-inflammatory, antiviral, antifungal, analgesic, antihyperglycemic, antimicrobial with antiparasitic activities and are useful in the prevention of inflammatory diseases.[28]

Acute toxicity studies
There was no record of death in the acute toxicity evaluation of aqueous extract of *C. limon* pith at a dose of 3000 mg/kg. Thus, the median lethal dose (LD_{50}) of the aqueous extract of *C. limon* pith was greater than 3000 mg/kg.

Effect of the aqueous extract of the pith of C. limon on cyclophosphamide-induced myelosuppression
The aqueous extract of *C limon* pith at doses 150, 300, 600 mg/kg significantly increased the white blood cells count and the platelets while a statistically (P<0.05) significant increase was observed in the neutrophils level at the extract dose of 150 mg/kg when compared to the negative control. Lymphocytes count was increased at 300 and 600 mg/kg doses of extract when compared to the cyclophosphamide group (Table 1). There was a statistically significant (P<0.05) increase in the monocytes level while the difference in eosinophils and erythrocyte sedimentation rate were non-significant (Table 2).
Cyclophosphamide was used as an immunosuppressive agent in this study to induce myelosuppression in the animals. The effect of *C. limon* on the haematological parameters of the rats indicated a significant increase in the white blood cells (WBC) count of the extract treated animals. The increase in WBC is suggestive of the ability of *C. limon* pith to stimulate the immune system leading to the production of white blood cells.[29]
This corroborates the findings of Kumuolosasi *et al*,[30] that *Mangifera indica* leaves and *Curcuma domestica* rhizomes methanol extract increased white blood cell counts and acted as potent immunostimulant agents.

Table 1: Effect of the aqueous extract of the pith of *C. limon* on cyclophosphamide-induced myelosuppression

Groups	Dose (mg/kg)	PCV (%)	Hb (g/dl)	RBC ($\times10^{12}$/L)	WBC ($\times10^9$/L)	Platelets ($\times10^9$/L)	Neutrophils (%)	Lymphocytes (%)
Control	2ml/kg	34.17 ± 1.19	12.5 ± 0.76	3.95 ± 0.15	215.67± 6.24	588.17± 18.07	27.67± 0.84	71.00 ± 1.18*
Cyclophosphamide	30	36.67 ± 1.67	12.3 ± 0.53	4.13 ± 0.17	192.00± 4.76	476.50± 26.48	34.16 ± 2.01	65.83 ± 2.01*
Levamisole	50	36.33 ± 1.33	12.22 ± 0.44	4.03 ± 0.15	183.67± 4.25*	609.67 ± 27.03	30.00 ± 0.52	69.17 ± 0.83
Extract	150	36.83 ± 1.19	12.33 ± 0.41	4.08 ± 0.14	217.67± 3.96*	598.00 ± 34.64	33.67± 2.23*	65.67 ± 1.94*
Extract	300	38.00 ± 1.61	12.82 ± 0.49	4.22 ± 0.19	224.17± 5.76*	612.83 ± 35.14*	30.50 ± 0.96	68.83 ± 0.83
Extract	600	34.33 ± 2.17	11.52 ± 0.73	3.83 ± 0.23	233.50± 7.28*	637.67± 28.00*	29.67 ± 0.61	68.83 ± 0.83

Data expressed as mean ± SEM, *P<0.05. PCV, Hb, RBC, WBC denotes packed cell volume, haemoglobin, red blood cell and white blood cell respectively.

Table 2: Effect of aqueous extract of *C. limon* pith on cyclophosphamide- induced myelosuppression

Groups	Dose (mg/kg)	Monocytes (%)	Eosinophils (%)	Basophils (%)	ESR (%)
Control	2 ml/kg	0.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.22
Cyclophosphamide	30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.17 ± 0.17
Levamisole	50	0.80 ± 0.54	0.00 ± 0.00	0.00 ± 0.00	0.83 ± 0.31
Extract	150	0.50 ± 0.34	0.17 ± 0.17	0.00 ± 0.00	0.67 ± 0.21
Extract	300	0.50 ± 0.34	0.50 ± 0.34	0.00 ± 0.00	0.67 ± 0.33
Extract	600	1.12± 0.40*	0.33 ± 0.21	0.00 ± 0.00	1.00 ± 0.37

Data expressed as mean ± SEM, *P<0.05. ESR denotes erythrocyte sedimentation rate.

Neeranuch and Sunthamala[31] in their study observed that the root, stem, and leaf crude extracts of *Maytenus mekongensis* increased white blood cells count and lymphocytes exhibiting immune enhancing properties which is similar to the results of the present study.

White blood cells are highly involved in phagocytosis and in enhancing defence against infection. Neutrophils are also involved in phagocytosis of bacteria. This also serves as a pointer to the immunostimulatory activity of *C. limon* pith. However, there was no significant difference in the packed cell volume, haemoglobin, and red blood cell levels in the treated animals when compared to the control. The lymphocytes play active roles in cellular and humoral immunity while the monocytes as monocyte–macrophages phagocytose bacteria including particulate material and are involved in inflammatory reactions through which they act on antigenic substances and attract T-lymphocytes in the immune system.[32]

Platelets level was significantly higher in the extract treated groups when compared to the controls. Platelets have been found to be involved in protection against microbial invasion, mobilization of innate immune cells and enhancement of their activity and influences antigen presentation thereby promoting adaptive immune responses.[33] The observed increase in the platelets is a signal to the presence of inflammation which is one of the ways a physiological system responds on exposure to substances such as injury, chronic diseases, or infections that challenge the immune system. Erythrocyte sedimentation rate serves as a measure of inflammatory activity in a biological system in some disease states such as tumors or infections or autoimmune disease.[34]

The erythrocyte sedimentation rate (ESR) is a common haematology test that may indicate and monitor an increase in inflammatory activity within the body caused by one or more conditions such as autoimmune disease, infections, or tumours.

Erythrocyte sedimentation rate (ESR) was non-significantly lower in the treated groups when compared to the untreated group. This indicates that the extract was able to offer some protection against the inflammatory immune cells.

Effect of C. limon pith extracts on delayed type hypersensitivity (DTH) response

There was an increase in paw thickness of rats injected with sheep red blood cells as antigen. It was observed that the percentage (%) increase in paw thickness increased with increase in extract dose when compared to the control (Table 3). Cell- mediated immunity is carried out through the activity of T-lymphocytes and their products (lymphokines). Delayed type hypersensitivity is usually evaluated by increase in foot paw thickness. Delayed type hypersensitivity response is an important aspect of the adaptive immune system of a biological system. It is often used to assay cell mediated immunity effects of medicinal plants in biological systems.[35]

The observed increase in the percentage of paw thickness exhibited by *C.limon* pith extract reflects the ability of the extract to stimulate inflammatory reaction which is an important component of immune response. This is like the report of Nfambi *et al*,[36] which stated that the methanolic leaf extract of *M. oleifera* caused a significant immunostimulatory effect on both the cell-mediated and humoral immune systems in the Wistar albino rats.

The sheep red blood cell used in this study was employed as a T-cell dependent antigen to provoke immune system response. Therefore, an increase in delayed type hypersensitivity reaction in rats in response to T- cell dependent antigen revealed the stimulatory effect of the aqueous extract of *C. limon* pith on T-cells.[37] The results suggest that the extract may have activated type 1 helper (Th1) cells and expanded antigen presenting cells (APCs) with class II major histocompatibility complex (MHC).[38] The activated T-cells might proliferate and release cytokines which will lead to increased vascular permeability, vasodilation, and macrophages accumulation resulting in increased phagocytic activity ultimately leading to inflammation.

The phytoconstituents of *C. limon* pith such as the flavonoids may be partly responsible for the observed immunostimulatory activities of *C. limon* pith as flavonoids have been widely reported to possess immune system enhancing properties and other biological activities.[39]

Table 3: Effect of *C. limon* pith extracts on delayed type hypersensitivity (DTH) response on rat paw

Groups	Dose (mg/kg)	Before Challenge	After Challenge	Increase in paw thickness (%)
Control	2ml/kg	4.35 ± 0.13	5.07 ± 0.14	17
Extract	150	4.32 ± 0.14	5.37 ± 0.12	24.3
Extract	300	4.10 ± 0.07	5.39 ± 0.07	31.5
Extract	600	4.23 ± 0.02	6.07 ± 0.02*	43

Data expressed as mean ± SEM, *P<0.05.

Conclusion

Based on the findings from the study, the aqueous extract of *C. limon* pith increased haematological parameters and cell-mediated immunity in Wistar rats which could be attributed to its phytochemical constituents. The aqueous extract of *C. limon* pith is immunostimulatory and therefore has a potential therapeutic value in immunosuppressing clinical conditions.

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.

References

1. Mahima AKV, Amit K, Vinod K, Anu R. Designer milk for human health: A future need. Int J Dairy Sci. 2012; 7 (3):70-72.
2. Okoye TC, Akah PA, Ezike AC, Uzor PF, Odoh UE, Igboeme SO, Onwuka UB, Okafor SN. Immunomodulatory effects of *Stachytarpheta cayennensis* leaf extract and its synergistic effect with artesunate. BMC Complement Altern Med. 2014; 5(14): 376.
3. Zumla A, Memish ZA, Maeurer M, Bates M, Mwaba P, Al-Tawfiq JA, Denning DW, Hayden FG, Hui DS. Emerging novel and antimicrobial-resistant respiratory tract infections: new drug development and therapeutic options. Lancet Infect Dis. 2014; 14(11):1136-1149.
4. Eze JI, Ndukwe S. Effect of methanol extract of *Mucuna pruriens* seed on the immune response of mice. Comparative Clin Pathol. 2012; 21:1343-1347
5. Efflein J. Coronavirus (Covid-19) cases, recoveries, and deaths worldwide. https://www.statista.com/statistics/1087466/covid19-cases-recoveries-deaths-worldwide/ (accessed January 6,2023).

6. Alhazmi HA, Najmi A, Javed SA, Sultana S, Al Bratty M, Makeen HA, Meraya AM, Ahsan W, Mohan S, Taha MME, Khalid A. Medicinal plants and isolated molecules demonstrating immunomodulation activity as potential alternative therapies for viral diseases including COVID-19. Front Immunol. 2021;13 (12) :637553.
7. Fong HH. Integration of herbal medicine into modern medical practices: issues and prospects. Integr Cancer Ther. 2002; 1(3):287-293; discussion 293. doi: 10.1177/153473540200100313. PMID: 14667286.
8. Yang Y, Islam MS, Wang J, Li Y, Chen X. Traditional Chinese medicine in the treatment of patients infected with 2019-new coronavirus (SARS-CoV-2): A review and perspective. Int J Biol Sci. 2020;16(10):1708-1717.:
9. Remali J, Aizat WMA. Review on plant bioactive compounds and their modes of action against coronavirus infection. Front Pharmacol. 2021;11: 1-13.
10. Liu M, Gao Y, Yuan Y, Yang K, Shi S, Zhang J, Tian J. Efficacy, and safety of integrated traditional Chinese and Western medicine for coronavirus disease 2019 (COVID-19): a systematic review and meta-analysis. Pharmacol Res. 2020; 8(158):104896. doi: 10.1016/j.phrs.2020.104896.
11. Ang L, Song E, Lee HW, Lee MS. Herbal Medicine for the Treatment of Coronavirus Disease 2019 (COVID-19): A Systematic review and meta-analysis of randomized controlled trials. J Clin Med. 2020; 23;9(5):1583.
12. Wu YQ, Zou L, Yu X, Sun D, Li SB, Tang L, Yang JR, Chen XY, Wu YG, Fang H. Clinical effects of integrated traditional Chinese and western medicine on COVID-19: a systematic review. Shanghai J Tradit Chin Med. 2020;1–8.
13. Mabberley DJ. Citrus (Rutaceae): A review of recent advances in etymology, systematics, and medical applications. Blumea J. Plant Taxon. Plant Geogr. 2004; 49:481–498.
14. Rafique S, Hassan SM, Mughal SS, Hassan SK, Shabbir N. Biological attributes of lemon: A review. J Addict Med Ther Sci. 2020; 6(1): 030-034.
15. Papp N, Bartha S, Boris G, Balogh L. Traditional uses of medicinal plants for respiratory diseases in Transylvania. Nat. Prod. Commun. 2011; 6:1459–1460.
16. Klimek-Szczykutowicz M, Szopa A, Ekiert H. *Citrus limon* (Lemon) Phenomenon-A Review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies. Plants (Basel). 2020; 17;9(1):119..
17. Makni M, Jemai R, Kriaa W, Chtourou Y, Fetoui H. *Citrus limon* from Tunisia: Phytochemical and physicochemical properties and biological activities. Biomed Res Int. 2018;1(15); 6251546. doi: 10.1155/2018/6251546. PMID:
18. Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction technologies for medicinal and aromatic plants. United Nations Industrial Development Organization and the International Centre for Science and High Technology, 2008, pp 23.
19. Trease GE, Evans WC. Text of Pharmacognosy. 13th Edition. Oxford University Press, London, United Kingdom. 1989; 21: 247, 304-309.
20. Organisation for Economic Cooperation and Development. Guidelines for testing of chemicals 425. Acute oral toxicity: Up and down procedure. 2008
21. Manjarekar PN, Jolly CL, Narayan S. Comparative studies of immunomodulatory activity of *Tinospora cordifolia* and *Tinospora sinensis*. Fitoterap. 2001; 71:254-257
22. Ochei J, Kolhatkar A. The complete blood counts. Medical laboratory science theory and practice. 2000 Tata McGraw-Hill Publishing Company Limited. pp.275–287.
23. Doherty NS. Selective effects of immunosuppressive agents against the delayed hypersensitivity response and humoral response to sheep red blood cells in mice. Agents Actions. 1981;11(3):237-242.
24. Jafarian-Dehkordi A, Zolfaghari B, Mirdamadi M. The effects of chloroform, ethyl acetate and methanolic extracts of *Brassica rapa* L. on cell-mediated immune response in mice. Res Pharm Sci. 2013;8(3):159-165.
25. Ravishankar D, Rajora AK, Greco F, Osborn HMI. Flavonoids as prospective compounds for anti-cancer therapy. Int J Biochem Cell Biol.2013; 45:2821-2831.
26. Urquiaga I, Leighton F. Plant polyphenol antioxidants and oxidative stress. Biol Res. 2000;33(2):55-64.
27. Hosseinzade A, Sadeghi O, Naghdipour Biregani A, Soukhtehzari S, Brandt GS, Esmaillzadeh A. Immunomodulatory effects of flavonoids: possible induction of T- CD4+ regulatory cells through suppression of mTOR pathway signaling activity. Front Immunol. 2019;10(1):1-12.
28. Franklin LU, Cunnington GD, Young D. Terpene based pesticide treatments for killing terrestrial arthropods including, amongst others, lice, lice eggs, mites and ants 2001 - Google Patents.
29. Taiwo IA, Oboh BO, Francis-Garuba PN. Haematological properties of aqueous extracts of *Phyllantus amarus* (Schum and Thonn.) and *Xylopia aethiopica* (Dunal) A. Rich in Albino rats. Ethno-Med. 2009; 3(2):99-103.
30. Kumolosasi E, Siti NAI, Siti MAS. Immunostimulant activity of standardized extracts of Mangifera indica leaf and Curcuma domestica rhizome in mice. Trop J Pharmaceut Res. 2018; 17(1):77-84.
31. Neeranuch S, Nuchsupha S. Phytochemical screening, Antioxidant activity and ex-vivo immunological-associated properties of a halophyte extract, *Maytenus mekongensis* In Sched. Trop J Nat Prod Res, 2021; 5(11):1949-1957
32. Blumenreich MS. The white blood cell and differential count. In: Walker HK, Hall WD, Hurst JW, editors. Clinical methods: The history, physical, and laboratory examinations. 3rd ed. Boston: Butterworths; 1990. Chapter 153. PMID: 21250104.
33. Ali RA, Wuescher LM, Worth RG. Platelets: essential components of the immune system. Curr Trends Immunol. 2015; 16:65-78.
34. Tishkowski K, Gupta V. Erythrocyte sedimentation rate. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan–. PMID: 32491417.
35. Khurana I. Textbook of medical physiology. 1st ed. India: Elsevier, 2005; pp 185-190
36. Nfambi J, Bhosa GS, Sembaiwe LF, Gakunga J, Kasolo JN. Immunomodulatory activity of methanolic leaf extract of *Moringa oleifera* in Wistar albino rats. J Basic Clin Physiol Pharmacol. 2015; 26(6): 603–611
37. Shah AS, Juvekar AR. Immunostimulatory activity of aqueous extract of *Murraya Koenigii* (Linn.) spreng leaves. Indian J Nat Prod Resour. 2010; 1(4):450-455.
38. Wieczorek M, Abualrous ET, Sticht J, Álvaro-Benito M, Stolzenberg S, Noé F, Freund C. Major histocompatibility complex (MHC) class I and MHC class II proteins: conformational plasticity in antigen presentation. Front Immunol. 2017; 8:292. doi: 10.3389/fimmu.2017.00292.
39. Anacleto SL, Lajolo FM, Hassimotto NM. Estimation of dietary flavonoid intake of the Brazilian population: a comparison between the USDA and phenol-explorer databases. J Food Compos Anal 78:1-8.