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Research article

In-vivo evaluation of immunomodulatory activity of crude extract and solvent fractions of *Cyphostemma adenocaule* (Steud. ex A.Rich)

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ABSTRACT

Background: Various indigenous medicinal systems including Ethiopians used *Cyphostemma adenocaule* for managing tumors, helminthiasis, snake envenomation, rabis virus, splenomegaly, and other immunological disorders. However, no scientific study was conducted to validate these traditional medicinal claims of the plant. Therefore, this study aimed to evaluate the *in-vivo* immunomodulatory activity of the crude root extract and its solvent fractions.

Methods: Carbon clearance rate and humoral antibody titer were determined for 100, 200, and 400 mg/kg of crude extract and solvent fractions among Swiss albino mice. Carbon ink and sheep red blood cells were used as antigens for carbon clearance assay and humoral antibody titer respectively.

Results: Among all groups, an increase in both carbon clearance rate and the humoral antibody titer was observed with an increase in the dose of both crude extract and solvent fractions. Compared to the solvent fractions of comparable doses, the crude extract showed better activity. The crude extract at a dose of 400 mg/kg revealed the highest and statistically significant augmentation of carbon clearance rate (0.1100 ± 0.0124) and humoral antibody titer (96.00 ± 14.31) compared to the vehicle control group.

Conclusion: From our study, it is concluded that crude extract and *n*-butanol fraction showed promising immunostimulant activity by enhancing carbon clearance rate and humoral antibody titer.

1. Introduction

A disturbance in the components of the immune system leads to the development of various health problems [1]. Immunocompromised patients are highly vulnerable to infectious diseases that occur following the entrance of microorganisms to host tissue [2]. Besides infection, cancer occurs when there is a loss of immunological control over actively dividing cells [3]. Protection from infectious or other immunological disorders is provided by the body's defensive capacity known as immunity [1]. This defensive capacity of the body is classified as innate immunity and adaptive immunity, Innate immunity is a broad-acting nonspecific immune response to any antigen developed by genetic constitutional makeup [4] whereas adaptive immunity is a specific immune response produced by a complex network of cells, genes, protein, and cytokines against a previously encountered antigen [5]. The adaptive immunity can be expressed in the form of humoral which is mediated by B lymphocyte-derived antibodies and cell-mediated immunity which is mediated by T lymphocytes [6].

Immunomodulation involves suppression or stimulation of the immune system to alter the associated immune response. The crude extract and isolates from different plant species have been reported to alter the immunological response to an antigenic matter. In the indigenous medicinal system of various cultures including Ethiopia, *C. adenocaule* has been reported to be used for the treatment of cancer, snakebite, malaria, rabies virus, and anthrax [7, 8, 9, 10]. Phytochemical investigation of the plant showed the presence of alkaloids, flavonoids, tannins, saponins, cardiac glycosides, carotenoids, xanthophyll, vitamin C, tocopherols, and tocotrienols [11, 12]. *C. adenocaule* is reported to for its antiplasmodial [13], antioxidant [11], anthelminthic [14], and anti-inflammatory activities [11]. The plant also has a proliferative effect on blood cells in mice models [15]. The traditional medicinal claims of the plant might be due to the immunomodulatory effect of its bioactive metabolites.

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Therefore, this study aimed to evaluate the immunomodulatory activity of crude extract and solvent fractions of *C. adenocaule* in mice using carbon clearance test and humoral antibody titer models.

2. Materials and methods

2.1. Chemicals, reagents, and standard drugs

For this study ethanol (Fine Chemical general trading, Addis Ababa, Ethiopia), *n*-hexane (Loba Chemie. Pvt. Ltd, India), *n*-butanol (CARLO ERBA Reagents SAS, France), gum acacia (Shreeji Pharma International, India), carbon ink (B. Dayal & Company Pvt. Ltd, India), levamisole (Ningbo Shuangwei Pharm.co.Ltd., China), cyclophosphamide (Cadila health care Ltd., India) were used. Laboratory/analytical grade chemicals, reagents, and standard drugs were used.

2.2. Plant material

Cyphostemma adenocaule root was collected from the *Angacha* riverside, North Gondar, Ethiopia. The plant was authenticated at the herbarium of biology department of the University of Gondar, Ethiopia. The voucher specimen with a reference number of DA01/2018 was deposited in the herbarium.

2.3. Experimental animal

Swiss albino mice of either sex aged 6–8 weeks and weighing 25–30g breed in the animal house of School of Pharmacy, College of Health Sciences, Mekelle University were used. The mice were acclimatized to the laboratory condition for a week before the experiment and handled throughout the experiment following the guideline for the care and use of laboratory animals [16].

2.4. Preparation of crude extract

In the traditional medicinal claim, medicinal practitioners use local liquor extract of the plant for the management of rabies virus [8]. Moreover, Study conducted by Feyisayo and his co-works reported that the ethanolic root extract of the plant revealed strong antioxidant activity than the stem bark extract [11]. Therefore, in this study, the extract was prepared from the root of the plant using ethanol as extraction solvent. The collected roots were dried under shade for two weeks and milled using a high-speed electric grinder. One thousand (1000) grams of the powder was macerated with five volumes (5000ml) of hydroethanolic solution (70%) for three days. After three days of maceration, the macerate was successively filtered with muslin cloth and Whatman filter paper No. 1 to retain the mark from the soluble component. The marc was re-macerated twice with the same solvent for three days [11]. The filtrates from all extraction cycles were combined and dried in an oven at 37 °C.

2.5. Solvent fractionation of the crude extract

The crude extract (60 g) was dispersed in 300ml of water in a separatory funnel and mixed by gentle shaking. To the aqueous dispersion 300ml of n-hexane was added and mixed by shaking. The mixture was allowed to settle for phase separation to get the upper *n*-hexane layer and lower aqueous layer. The process was repeated in triplicates to get the n-hexane fraction. After collection of the n-hexane fraction, the aqueous layer was again treated with 300ml *n*-butanol in triplicates to obtain the *n*-butanol fraction and the remaining aqueous residue. The three fractions were separately dried in an oven at 37 °C [11].

2.6. Sheep red blood cell (SRBC) preparation and standardization

Sheep blood was withdrawn from the external jugular vein in a 1:1 proportion of freshly prepared Alsever's solution. SRBCs were separated from the collected blood by centrifuging at 2500 rpm for 10min and washed with pyrogen-free normal saline (0.9% w/v). The concentration was adjusted to 1×10^8 SRBC in 1 mL of sheep blood [17, 18].

2.7. Preparation of carbon ink suspension

Carbon ink diluted with eight volumes of normal saline was employed as antigenic material for the determination of the phagocytic activity [19].

2.8. Vehicle and standard drug selection

The hydroethanolic extract is not soluble in water and polysorbates (tween 80)have immunomodulatory property [20, 21] the doses for the study were prepared in suspension form using 2% gum acacia as a suspending agent. Levamisole is used as a standard immunostimulant drug due to its ability to enhance or restore both humoral and cellular immune responses in humans and animals [22]. Furthermore, levamisole is selected due to its local availability in our country. Cyclophosphamide was used as a standard immunosuppressant drug due to its antimitotic effect. Besides, cyclophosphamide causes selective suppression of T-cell mediated immune response [23].

2.9. Animal grouping, dosing, and immune response studies

Based on the reported safety profile of the crude extract and solvent fractions at a limit dose of 2000 mg/kg; 100, 200, and 400 mg/kg of crude extract and solvent fractions were taken as treatment doses [15]. Thus, Mice were randomly grouped into sixteen groups for the carbon clearance test (Table 1) and twelve groups for Humoral antibody titer (Table 2). Each group consist of six mice that received respective vehicle/standard drug/extract/solvent fractions Thus, a total of 168 mice (96 for carbon clearance test and 72 for Humoral antibody titer test) were used for the study. Due to its negligible effect in the carbon clearance test, n-hexane fraction was not tested for its effect in Humoral antibody titer to reduce wastage of experimental animals. Doses were freshly prepared as homogenized suspension of crude extract and solvent fractions (aqueous and *n*-butanol fraction) in 2% gum acacia. However, the *n*-hexane fraction was dissolved in 10 mL/kg sunflower oil [24].

2.9.1. Carbon clearance test

To evaluate the phagocytic activity of the reticuloendothelial system, mice were grouped based on the above protocol (Table 1) and received

Table 1. Grouping and dosing for Carbon clearance test.

Group	N	Dose
I	6	10 mL/kg VH
II	6	50 mg/kg LEV
III	6	30 mg/kg CYP
IV	6	100 mg/kg CE
V	6	200 mg/kg CE
VI	6	400 mg/kg CE
VII	6	100 mg/kg AF
VIII	6	200 mg/kg AF
IX	6	400 mg/kg AF
X	6	100 mg/kg BF
XI	6	200 mg/kg BF
XII	6	400 mg/kg BF
XIII	6	100 mg/kg HF
XIV	6	200 mg/kg HF
XV	6	400 mg/kg HF
XVI	6	10 mL/kg SO

N, number of mice per group, VH = vehicle (2% gum acacia), LEV = levamisole, CYP = cyclophosphamide, CE = Crude extract, AF = aqueous fraction, BF = *n*-butanol fraction, HF = *n*-hexane fraction, SO = sunflower oil.

Table 2. Grouping and dosing for Humoral antibody titer.

Group	N	Dose
I	6	10 mL/kg VH
II	6	50 mg/kg LEV
III	6	30 mg/kg CYP
IV	6	100 mg/kg CE
V	6	200 mg/kg CE
VI	6	400 mg/kg CE
VII	6	100 mg/kg AF
VIII	6	200 mg/kg AF
IX	6	400 mg/kg AF
X	6	100 mg/kg BF
XI	6	200 mg/kg BF
XII	6	400 mg/kg BF

N, number of mice per group, VH = vehicle (2% gum acacia), LEV = levamisole, CYP = cyclophosphamide, CE = Crude extract, AF = aqueous fraction, BF = *n*-butanol fraction.

the respective treatment for 5 days. After 48 h of the last dose, all groups of mice received 10 mL/kg carbon ink suspension intravenously through the tail vein. Blood was withdrawn from the retro-orbital plexus of mice at 5 and 15 min after injection of the carbon suspension. The collected blood (25 μL) was lysed with 3 mL of 0.1% sodium carbonate and optical density was determined by measuring the absorbance at 675 nm using 0.1% sodium carbonate as a blank solution. The clearance rate of carbon particles (*K*) through phagocytosis by the reticuloendothelial system was determined from optical densities (equation [1]) [18, 25]. The percentage change (equation 2) in the mean carbon clearance rate indicates the immunomodulatory property of both the crude extract and solvent fractions compared to the normal control group [26].

$$K = \frac{\ln OD1 - \ln OD2}{(t2 - t1)} \quad (1)$$

$$\% \text{ Change} = \frac{(K\text{treatment} - K \text{ control}) \times 100}{K \text{ treatment}} \quad (2)$$

Where, K: carbon clearance rate by the reticuloendothelial system.

OD1: Optical density at a time (t1) 5 min after ink injection to the tail vein.

OD2: Optical density at a time (t2) 15 min after ink injection to the tail vein.

2.9.2. Humoral antibody titer (HAT) response to SRBC

To determine the humoral response to SRBC all mice were immunized on day 0 with an intraperitoneal injection of 0.1 mL of SRBC suspension containing 1×10^8 cells/mL and grouped according to the aforementioned protocol (Table 2). Treatment started after 24 h of immunization and continued for 14 days. On day 7, 1 h after dose administration, all groups of mice were challenged with an intraperitoneal injection of 0.1 mL of SRBC suspension in normal saline containing 1×10^8 cells/mL. On the 14th day, blood was withdrawn from the retro-orbital plexus of all mice and centrifuged at 2500 rpm for 10 min to separate the serum [27]. The HAT test was conducted according to the method described by puri *et al* (1994). The percentage change in HAT compared to the control was determined (equation 3) [28].

$$\% \text{ Change} = \frac{(\text{HAT treatment} - \text{HAT control}) \times 100}{\text{HAT treatment}} \quad (3)$$

2.10. Data analysis

The results were analyzed using Statistical Package for Social Science (SPSS) version 20 Software and presented as Mean ± SEM (Standard Error of Mean). The statistical difference between groups was determined by using one-way ANOVA followed Tukey post hoc multiple comparison test to compare the level of alteration in the carbon clearance rate and humoral antibody titer. These results were considered significant when the *p*-value <0.05 at a 95% confidence interval.

2.11. Ethical consideration

All the experiments were conducted following internationally accepted laboratory animal use and care guidelines. All the experiments were started after approval of study protocol and ethical issues by the Health Research Ethics Review Committee (HRERC) of Mekelle University and given an ethical clearance code of 1548/2018 for its compliance with the principle of experimental animal use.

3. Result and discussion

As presented in the table below (Table 3), the crude extract and solvent fractions of *C. adenocaule* increased the clearance rate of carbon particles compared to the vehicle (2% gum acacia) and cyclophosphamide treated groups. This enhancement of carbon clearance rate indicated stimulation of the reticuloendothelial system which is responsible for the phagocytosis of carbon particles. Antioxidants promote the function of the immune system [29, 30]. Hence, the increased carbon clearance activity of the plant might be attributed to its antioxidant activity [11] owing to the presence of antioxidant phenolic compounds [11, 31].

The increased clearance rate might also be attributable to stimulation of the hematopoietic system and enhanced the differentiation of lymphocytes to B and T-lymphocyte subtypes that produce an antibody and

Table 3. The effect of the controls, crude extract, and solvent fractions of *Cyphostemma adenocaule* on carbon clearance rate and humoral antibody titer.

Groups	Dose (mg/kg)	CCR (mean ± SEM)	Change (%)	Lu HAT (mean ± SEM)	Change (%)
VH	-	0.0413 ± 0.0053	-	32.00 ± 7.16	-
LEV	50	0.1377 ± 0.0080^{a***c***g*}	↑ 70.01	80.00 ± 16.00 ^{c**}	↑ 60.00
CYP	30	0.0357 ± 0.0079	↓ 15.69	21.33 ± 3.37	↓ 50.00
CE	100	0.0800 ± 0.0135	↑ 48.38	58.67 ± 5.33	↑ 45.46
	200	0.1087 ± 0.0085^{a*c**}	↑ 62.01	74.67 ± 10.67^{c*}	↑ 57.14
	400	0.1313 ± 0.0080^{a***c***g*}	↑ 68.55	96.00 ± 14.31 ^{a**c***}	↑ 66.67
AF	100	0.0684 ± 0.0236	↑ 39.62	53.33 ± 6.75	↑ 40.00
	200	0.0889 ± 0.0190	↑ 53.54	64.00 ± 0.00	↑ 50.00
	400	0.1100 ± 0.0124^{a*c**}	↑ 62.45	74.67 ± 10.67^{c*}	↑ 57.14
BF	100	0.0795 ± 0.0212	↑ 48.05	53.33 ± 6.75	↑ 40.00
	200	0.1145 ± 0.0074^{a**c**}	↑ 63.93	69.33 ± 12.84	↑ 53.84
	400	0.1221 ± 0.0046^{a***c***}	↑ 66.18	85.33 ± 13.49 ^{a*c**}	↑ 62.50
SO	-	0.0481 ± 0.0090	-		
HF	100	0.0494 ± 0.0114	↑ 2.63		
	200	0.0499 ± 0.0087	↑ 3.61		

Results were expressed as Mean ± SEM; n = 6. Abbreviations: SEM: Standard error of mean, VH = vehicle, LEV = Levamisole, CYP = Cyclophosphamide, CE = crude extract, AF = Aqueous fraction, BF = *n*-Butanol fraction, HF = *n*-Hexane fraction, SO = Sunflower oil, CCR: Carbon clearance rate, HAT = humoral antibody titer, ↑ = Potentiation, and ↓ = suppression, ^{a} = compared to gum acacia, ^{c} = compared to cyclophosphamide, and ^{g} = compared to100 mg/kg aqueous fraction, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

INF-γ molecules respectively [5, 32]. Among the test groups, 400 mg/kg of the crude extract revealed the highest carbon clearance rate (0.1313 ± 0.0080) stimulating the clearance rate by 68.55% compared to the vehicle (Table 3). The higher clearance rate exhibited by the crude extract relative to solvent fractions of the corresponding dose could be due to the presence of various secondary metabolites at a higher concentration that may additively or synergistically boost phagocytic function [33]. Among the solvent fractions, the *n*-butanol fraction showed the highest stimulation of carbon clearance rate. However, the *n*-hexane fraction of *C. adenocaule* revealed a minimal and statistically insignificant increase in the mean clearance rate of carbon particles compared to sunflower oil which might be attributable to negligible concentration of immunostimulant phytochemicals of high to medium polarity in the *n*-hexane fraction [34].

As presented in the above table (Table 3), the crude extract and solvent fractions of *C. adenocaule* at all doses increased the level of secondary HAT to SRBC compared to the vehicle. This augmentation of HAT values indicated stimulation of antibody production against SRBC antigen. The crude extract at a dose of 400 mg/kg induced the maximum (96.00 ± 14.31) statistically significant ($p < 0.01$) mean secondary HAT to SRBC enhancing the level of HAT by 66.67% compared to the vehicle. This augmentation might be attributable to a higher concentration of metabolites that strongly stimulate the proliferation of blood cells that are involved in humoral immunity. Among the solvent fractions, the *n*-butanol fraction of *C. adenocaule* at a dose of 400 mg/kg exhibited the highest enhancement in secondary HAT (85.33 ± 13.49) compared to the aqueous fraction of a comparable dose. This might be due to the difference in the nature and concentration of phytoconstituents present in the two solvent fractions. Compared to the vehicle control group, the highest dose of *n*-butanol fraction augmented the level of HAT to SRBC by 62.50%. The enhancement of HAT might be accredited to the presence of flavonoids, terpenoids [35], saponins, tannins, carbohydrates, alkaloids, and proteins which stimulate the production of macrophages, B and T lymphocytes [1, 36]. This finding is consistent with the finding of Feyisayo and Oluokun which showed the antibody precipitating capacity of a polysaccharide fraction of *C. adenocaule* in the agar well diffusion method [37]. It is also in line with the study of Ojogbane and his coworkers that reported stimulation of HAT by aqueous extract of *C. glaucophilla* leaves [38].

4. Conclusion

The crude extract and solvent fractions of *C. adenocaule* had immunostimulatory properties. This immunostimulant property of the plant could be due to its ability to increase the number of activated immune cells responsible for humoral and nonspecific immune responses. Therefore, the results of the study support the use of the plant by traditional healers for the management of envenomation, infections, and tumor.

5. Recommendation

From the current finding, the authors recommended the sub-acute and chronic toxicological studies on the crude extract and solvent fractions to determine the long-term safety profile of the plant. The authors also recommended the identification and characterization of compounds present in the more active *n*-butanol fraction to determine the active constituents responsible for its immunomodulatory effects. Besides, further studies should be carried out to reveal the mechanism of immunostimulation at a molecular level.

Declarations

Author contribution statement

Dehnnet Abebe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Aman Karim: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Helen Bitew: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Gomathi Periasamy: Analyzed and interpreted the data; Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

[1] P. Brindha, Role of phytochemicals as immunomodulatory agents: a review, Int. J. Green Pharm. 10 (1) (2016) 1–18.
[2] A.K. Abbas, A.H. Lichtman, S. Pillai, Cellular and molecular immunology, in: ELSEVIER SAUNDERS, 2012.
[3] J. Owen, J. Punt, S.A. Stanford, Cells, organs, and microenvironments of the immune system, in: Kuby Immunology, seventh ed., Susan Winslow, New York, 2013, pp. 27–41.
[4] WdM. Cruvinel, D. Mesquita Junior, J.A.P. Araujo, et al., Immune system: Part I. Fundamentals of innate immunity with emphasis on molecular and cellular mechanisms of inflammatory response, Rev. Bras. Reumatol. 50 (2010).
[5] P.J. Delves, S.J. Martin, D.R. Burton, I.M. Roitt, Innate immunity, in: Roitt's Essential Immunology 67, John Wiley & Sons, UK, 2017, pp. 40–54.
[6] A. Gokhale, A. Damre, M. Saraf, Investigations into the immunomodulatory activity of Argyreia speciosa, J. Ethnopharmacol. 84 (1) (2003) 109–114.
[7] S. Araya, B. Abera, M. Giday, Study of plants traditionally used in public and animal health management in Seharti Samre District, Southern Tigray, Ethiopia, J. Ethnobiol. Ethnomed. 11 (1) (2015) 1–25.
[8] Z. Birhanu, A. Endale, Z. Shewamene, An ethnomedicinal investigation of plants used by traditional healers of Gondar town, North-Western Ethiopia, J. Med. Plants Stud. 3 (2) (2015) 36–43.
[9] P. Nalumansi, M. Kamatenesi-Mugisha, G. Anywar, Medicinal plants used in paediatric health care in Namungalwe sub county, Iganga district, Uganda, Nova J. Med. Biol. Sci. 2 (3) (2014) 1–14.
[10] D.O. Ochwangi, C.N. Kimwele, J.A. Oduma, P.K. Gathumbi, J.M. Mbaria, S.G. Kiama, Medicinal plants used in treatment and management of cancer in Kakamega County, Kenya, J. Ethnopharmacol. 151 (3) (2014) 1040–1055.
[11] A. Feyisayo, A.V. Oluwafemi, O.O. Oluokun, Evaluation of antioxidant capacity and membrane stabilizing potential of stem and root of Cyphospenna adenocaulis (Steud), Afr. J. Biotechnol. 14 (21) (2015) 1820–1827.
[12] M. Yemele, P. Telefo, L. Lienou, et al., Ethnobotanical Survey of Medicinal Plants Used for Pregnant Women′ S Health Conditions in Menoua Division-West Cameroon 160, 2015, pp. 14–31.
[13] J.R. Chouna, F. Nardella, B.N. Lenta, C. Vonthron-Senecheau, P. Nkeng-Efouet-Alango, N. Sewald, Ceanothane-type triterpenoids from Cyphostemma adenocaule, Arch Pharm. Res. (Seoul) (2016) 1–6.
[14] O.M. Bello, S.M. Jagaba, O.E. Bello, et al., Phytochemistry, pharmacology and perceived health uses of non-cultivated vegetable Cyphostemma adenocaule (Steud. Ex A. Rich.) Desc. ex Wild and RB Drumm: a review, J. Scient. Afr. 2 (2019), e00053.

[15] D. Abebe, G. Periasamy, A. Karim, H. Bitew, Evaluation of the effect of hydroethanolic root extract and solvent fractions of Cyphostemma adenocaule (Steud. Ex A. Rich) descoings ex wild & drummond on cell-mediated immune response and blood cell count in mice, Evid. base Compl. Alternative Med. 2021 (2021).
[16] National Research Council, Guide for the Care and Use of Laboratory Animals, eighth ed., 2010.
[17] L. Ahirwal, S. Singh, M.K. Dubey, V. Bharti, A. Mehta, S. Shukla, In vivo immunomodulatory effects of the methanolic leaf extract of Gymnema sylvestre in Swiss albino mice, Arch. Biol. Sci. 67 (2) (2015) 561–570.
[18] P. Shrestha, M. Handral, P. Sciences, Evaluation of immunomodulatory activity of extract from rind of Nephelium lappaceum fruit, Int. J. Pharm. Pharmaceut. Sci. 9 (1) (2017) 38–43.
[19] N. Dashputre, N. Naikwade, Immunomodulatory activity of Abutilon indicum linn on albino mice, J. Int. J. Pharm. Sci. Res. 1 (3) (2010) 178–184.
[20] S.N. Al-Thamir, M.A. Al-Saadi, I.O. Jebur, Investigation the immunoadjuvant activity for polysorbate 80, Asian J. Pharm. Nurs. Med. Sci. 1 (1) (2013).
[21] J.B. Barnett, Immunosuppressive effects of tween 80 on mice, Int. Arch. Allergy Appl. Immunol. 66 (2) (1981) 229–232.
[22] O. Oladele, B. Emikpe, C. Adeyefa, F. Enibe, Effects of levamisole hydrochloride on cellular immune response and flock performance of commercial broilers, Brazil. J. Poult. Sci. 14 (2012) 259–265.
[23] M. Ahlmann, G. Hempel, The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy, Cancer Chemother. Pharmacol. 78 (4) (2016) 661–671.
[24] S.A. Farr, M.L. Niehoff, M.A. Ceddia, et al., Effect of botanical extracts containing carnosic acid or rosmarinic acid on learning and memory in SAMP8 mice, Physiol. Behav. 165 (2016) 328–338.
[25] S. Joghee, Immunomodulatory activity of ethanolic extract of artemisia abrotanum, Int. J. Pharmacogn. Phytochem. Res. 7 (3) (2015) 390–394.
[26] S. Singh, C. Yadav, M.N. Noolvi, Immunomodulatory activity of butanol fraction of Gentiana olivieri Griseb. on Balb/C mice, Asian Pac. J. Trop. Biomed. 2 (6) (2012) 433–437.
[27] D. Kanjwani, T. Marathe, S. Chiplunkar, Sathaye, Evaluation of immunomodulatory activity of methanolic extract of Piper betel, Scand. J. Immunol. 67 (6) (2008) 589–593.
[28] A. Puri, R. Saxena, R. Saxena, K. Saxena, V. Srivastava, J. Tandon, Immunostimulant activity of Nyctanthes arbor-tristis L, J. Ethnopharmacol. 42 (1) (1994) 31–37.
[29] A. Bendich, Physiological role of antioxidants in the immune system, J. Dairy Sci. 76 (9) (1993) 2789–2794.
[30] S. Hajian, Positive effect of antioxidants on immune system, Immunopathologia Persa 1 (1) (2014) e02.
[31] J.C.N. Assob, A.J. Njouendou, P.E. Nkeng, et al., In vitro screening of antioxidant properties of ten Cameroonian medicinal plants, J. Adv. Biotechnol. 3 (2) (2014).
[32] A. Ganeshpurkar, A.K. Saluja, Experimental animal models used for evaluation of potential immunomodulators: a mini review, Bull. Faculty Pharm. 55 (2) (2017) 211–216.
[33] E.M. Williamson, Synergy and other interactions in phytomedicines, Phytomedicine 8 (5) (2001) 401–409.
[34] S. Gabhe, P. Tatke, T. Khan, Evaluation of the immunomodulatory activity of the methanol extract of Ficus benghalensis roots in rats, Indian J. Pharmacol. 38 (4) (2006) 271.
[35] T. Raphael, G. Kuttan, Effect of naturally occurring triterpenoids glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin on the immune system, J. Phytomed. 10 (6-7) (2003) 483–489.
[36] F. Sharififar, S. Pournourmohammadi, M. Arabnejad, Immunomodulatory activity of aqueous extract of Achillea wilhelmsii C. Koch in mice, Indian J. Exp. Biol. 47 (2009) 668–671.
[37] A.K. Feyisayo, O.O. Oluokun, Isolation, fractionation, chemical constituents and activities of polysaccharides of stem and root of Cissus adenocaulis (Steud), J. Med. Plants Res. 7 (43) (2013) 3211–3219.
[38] O. Eleojo, O. James, N. Chiletugo, Effects of aqueous extract of" Cyphostemma glaucophilla" leaves on Some specific and non-specific immune responses in albino rats, Br. J. Pharmacol. Toxicol. 4 (4) (2013) 142–146.