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EVALUATION OF MIXTURES OF AQUEOUS EXTRACT OF *PSEUDOCEDRELA KOTSCHYI* AND *BOSWELLIA DALZEILII* AS INTERACTING PRODUCTS IN REGULATION OF PHAGOCYTOSIS AND LYMPHOCYTES PROLIFERATION

Oumar Mahamat [*1, 3], Tume Christopher [2], Kamtchueng Monique Odette [2] and Kamanyi Albert [3]

Department of Biological Sciences [1], Faculty of Science, University of Bamenda, Cameroon.
Department of Biochemistry [2], Department of Animal Biology [3], Faculty of Science, University of Dschang, Cameroon.

Key words:

Aqueous extracts, Macrophages, Lymphocytes, antogonism, *Synergy, Boswellia dalzeilii; Pseudocedrela kotschyi*

Correspondence to Author:
Oumar Mahamat

Department of Biological Sciences, Faculty of Science, University of Bamenda, Cameroon.

E-mail: oumar.mahamat@yahoo.fr

ABSTRACT: The current study aimed to test the effect of the aqueous extract of *Boswellia dalzeilii* and *Pseudecedrela kotschyi* using three different mixing ratios (*B. dalzeilii:P. kotschyi*) 3:1, 2:2, and 1:3. The assays concerned the study of the interactions in the modulation of phagocytic index, nitric oxide production, lysosomal enzymes and myeloperoxydase activity in macrophages and regulation of lymphocytes proliferation. The study results showed that the extracts of these plants exhibited antagonistic effects in the phagocytic index and production of NO while they produced synergetic effects in the stimulation of MPO-dependent activity and lymphocytes proliferation. Antagonistic effects were produced in the ratios 3:1 and 2:2 and synergetic properties in the ratios 1:3 for the stimulation of the activities of the lysosomal enzymes. These results look quite interesting and important and merit further investigations to support the present findings.

INTRODUCTION: From a historical perspective, medicines production and the pharmacological treatment of diseases started with the use of medicinal plants [1]. In many cases of therapy the people use two or more products or plants. The reasons for that are multiples; one hypothesis is synergistic interaction or multi-factorial effects between compounds present in herbal extracts. On the other hand, the pure drugs produced or isolated from these plants have rarely the same degree of activity as the unrefined extract at comparable concentrations or dose of the active component.

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This phenomenon is attributed to the absence of interacting substances [2]. In Cameroon, use of plants decoction obtained from combination of the barks of B. dalzeilii and *P. kotschyi* is promoted as an alternative treatment for some diseases associated to immunological disorders. In a recent study, it was demonstrated that extracts were obtained from dry barks of *B. dalzeilii* using water; methanol and hexane have a potent immunomodulatory activities *in vitro* test [3].

In the study we screen the interactions between aqueous extracts of *B. dalzeilii* and *P. kotschyi* which may explain if mixing crude extracts are a valid complementary approach for improving the immune response as are demonstrated by the extracts separately. Different formulations of the mixtures (3:1, 1:1 and 1:3) were tested on some biological activity related the actions of lymphocytes and macrophages.

MATERIALS AND METHODS:

Plant extract:

Collection of plant material:

The whole trunk barks of the plant *P. kotschyi* (Schweinf) Harms (family: Meliaceae) and *B. dalzeilii* (Hutch) Harms (family: Meliaceae) were collected in August in North region of Cameroon. These plants are been authenticated at the National Herbaruim, Yaounde, Cameroon where have been registered under the number 4359/SRFK and 7009/SRF/Cam respectively. The barks of trunk were cut and dried out of sun light. After drying, the barks were powered by using a laboratory blender. The fine powder was used for extraction using water distilled.

Preparation of extracts:

The extracts were extracted by boiling 500 g of the powdered barks in 1500 ml distilled water for 20 min. the filtrate obtained after filtration using No 3 Wahtman paper were dried in a vacuum desicator to obtain the crude aqueous extracts. The prepared extract was weighted and stored at 4°C.

Mononuclear cells:

Lymphocyte isolation from blood:

A blood sample (10 ml) collected in a sterile glass tube containing a drop of preservative-free heparin (500 IU Leo). It was allowed to stand for 2hrs at 37°C and the plasma rich in white blood cells was used for mononuclear cell purification. The cells were separated and isolated by density-gradient centrifugation on Ficoll-hypaque [4]. The mononuclear cells constituted of lymphocytes and monocytes were collected at the interface plasma-Ficoll hypaque. The cell number was counted with a haemocytometer using the trypan blue dye exclusion technique.

Monocytes and generation of macrophages:

Monocytes were separated from lymphocytes by adhesion on plastic support [5]. Monocytes at a density of $5x10^{10}$cells/ml were cultured in the presence of granulocyte macrophage colony-stimulating factor (40ng/ml) in culture medium (RMPI-1640) containing 5% foetal calf serum (FCS) and penicillin / streptomycin 100 U/ml at 37°C to differentiate into macrophages after on day 7.

Immunological assays:

Phagocytosis assays:

Using macrophages, extracts were screened for their potential to enhance phagocytosis. The phagocytosis assays were carried out by using the Neutral Red [6]. Assays were run in 96-well microculture plates. In order to stimulate phagocytosis in responses to CGB (Calmette Guerin bacillus), extracts were added at various concentrations from 160 to 2560 µg/ml. After incubation at 37°C, 0.075% neutral red solution was added to all wells for 1h prior to adding cell lysate solutions (ethanol and 0.001% acetic acid at the ratio of 1:1). Using an ELISA reader, the absorbance value at 492 nm was quantitated. The effect of extract was evaluated by calculating the phagocytic index or stimulatory activity of the extracts was calculated against control (F_1).

$$\textbf{(F}_1\textbf{) Phagocytic index } (\%) = \frac{[ODsample - ODcontrol]}{ODcontrol} x100$$

Nitric oxide production assay:

Supernatant from the previous macrophages culture (phagocytosis assays) was collected. Nitrite accumulation, an indicator of nitric oxide production, was measured in the supernatant using the Griess reagent [3]. The activity of the extract was evaluated against spontaneous production (**F_2**) and compared to activity of CBG alone.

$$\textbf{(F}_2\textbf{) Extract Activity } (\%) = \frac{[Conc.sample - Conc.control]}{Conc.control} x100$$

Measurement of metabolic activity in macrophages

Study of metabolic activity was based on the measurement of lysosomal enzyme and myeloperoxidase activity. In order to evaluate the lysosomal enzyme and myeloperoxidase activity, a double assay was done using *p*-nitrophenyl phosphate and *O*-phenylenediamine respectively [3]. After incubation for 24h, to measure the cellular lysosomal enzyme, 0.1% Triton X-100 (25 µl) were

added for 30 min prior to adding 10 mM *p*-nitrophenyl phosphate (150 µl) followed by 0.1 M citrate buffer (50 µ l, pH 5.0). The reaction was stopped using 0.2 M borate buffer (50 µ l, pH 9.8) after incubation for 1h at 37°C. The MPO-dependent activity was evaluated using 20 µl of a mixture of *o*-phenylenediamine (0.4 g/ml) and 0.002% H_2O_2 in phosphate-citrate buffer (v/v, pH 5.0). The reaction was stopped after 10 min using 0.1 N H_2SO_4. At the end of the reactions, the optical densities were measured at 490 nm and 405 nm for the lysosomal enzyme and myeloperoxidase activity respectively and the activity of the extracts were calculated **(F_3).**

$$(\boldsymbol{F_3})\ \boldsymbol{Activity}\ (\%) = \frac{[ODsample - ODcontrol]}{ODcontrol} x100$$

Measurement of antigen-specific cellular proliferation: Proliferation assays were performed concurrently with the lymphocytes to evaluate T and B cells responses. Assays were run in 96-well microculture plates, using lymphocytes. In order to stimulate proliferation inducing by Ag: PHA (4 µg/ml) and LPS (1 µg/ml), extracts were added at various concentrations from 160 to 2560 µg/ml. After incubation at 37°C, MTT was added to all wells prior 4h to adding isopropyl. Cellular proliferation was quantitated by reading the absorbance values at 492 nm using an ELISA reader. The effect of extract was calculated as the ratios of the experimental values to the value of control divided by the control.

Data analysis: The results were collected after 48 hours, % plant activity calculated against control treated with antigen only. Extract values of EC_{50} was analyzed by probit analysis using the maximum likelihood estimation [7, 8]. The association coefficient per EC-Extract mixture was used to determine their responses. If a mixture (M) compounds of two parts (A and B), and both components have EC_{50}, then the formulas below are used (A serving as standard) [9].

In this study *P. kotschyi* was considered as products A and *B. dalzeilii* as product B. Then, the EC_{50} of *P. kotschyi* or ConA was considered as standard and its efficiency index (Ea) is supposed equal to 100. The two extracts have the antagonistic effect when the association coefficient is less than 1 and synergistic when it was higher than 1 [10].

$$(\mathbf{F_4})\ Eb = \frac{ConA}{ConB} x100;$$

$$(\mathbf{F_5})\ Actual...Em = \frac{ConA}{ConM} x100,$$

Eb and E_m are the efficiency index of *B. dalzeilii* and the mixture respectively.

$(\boldsymbol{F_6})\ Theoretical.Em = (Ea \times \%A) + (Eb \times \%B)$, %A and %B are the percentage of *P. kotschyi* and *B. dalzeilii* in the mixture. Finally the synergistic factor or association coefficient was:

$$(\mathbf{F_7})\ Synergistic.factor = \frac{Actual..Em}{Theoretical..Em} x100$$

If one component of the mixture alone (for example B) had not determined because of high effect at all doses (>100%), the association coefficient of the mixture was calculated as followed:

$$(\boldsymbol{F_8})\ Synergistic.factor = \frac{Con..A}{Con..M} x100$$

RESULTS AND DISCUSION:

Activity of *B. dalzeilii*: In the immunological tests with the extract of *B. dalzeilii* the EC_{50} value has been calculated as 1206, 876.83, 59.14, 46742, 146.80 and 383.80 $\mu g.\mu l^{-1}$ for Phagocytic Index, NO Production, Lysosomal Enzymes Activity, Myeloperoxidase Activity, T-dependent lymphocytes proliferation and T-independent lymphocytes proliferation respectively. Here the extract of *B. dalzeilii* was more efficient in stimulation of Lysosomal Enzymes Activity followed by effect on NO Production, Phagocytic Index and Myeloperoxidase Activity of macrophages. The extract was also more active in stimulation of proliferative response of lymphocytes T than B cells (**Table 1**).

TABLE 1: EC_{50}, 95% CONFIDENCE LIMITS AND PROBABILITY OF AQUEOUS EXTRACT OF *B. DALZEILII* TO SOME IMMUNOLOGICAL ACTIVITY AFTER 48H OF INCUBATION

Cells	Immunological Activity	EC_{50} ($\mu g.\mu l^{-1}$)	95% Confidence Limits		X^2 (dl)	Probability
			Lower ($\mu g.\mu l^{-1}$)	Upper($\mu g.\mu l^{-1}$)		
Macrophages	Phagocytic Index	1206	873.6	1858	5.82 (3)	0.120
	NO Production	876.83	691.01	1154	5.79 (3)	0.12
	Lysosomal Enzymes Activity	59.14	-	-	-	-
	Myeloperoxidase Activity	46742	11727	3678573	3.39 (3)	0.33
Lymphocytes	Proliferation PHA-dependent	146.80	9.36	200.68	-	-
	Proliferation LPS-dependent	383.80	-	-	13.51 (2)	0.001

Activity of *P. kotschyi*:

With the extract of *P. kotschyi* the EC_{50} value has been calculated as 8091, 308.63, 284, 3423, 217.50 and 1018 $\mu g.\mu l^{-1}$ in stimulation of Phagocytic Index, NO Production, Lysosomal Enzymes Activity, Myeloperoxidase Activity, T-dependent lymphocytes proliferation and T-independent lymphocytes proliferation respectively. The extract effect on the macrophages was more important on the Lysosomal Enzymes Activity followed by effect on NO Production, Phagocytic Index and Myeloperoxidase Activity. On lymphocytes proliferation, the extract was more active in presence T-dependent antigen than T-independent antigen (**Table 2**).

TABLE 2: EC_{50}, 95% CONFIDENCE LIMITS AND PROBABILITY OF AQUEOUS EXTRACT OF *P. KOTSCHYI* TO SOME IMMUNOLOGICAL ACTIVITY AFTER 48H OF INCUBATION

Cells	Immunological Activity	EC_{50} ($\mu g.\mu l^{-1}$)	95% Confidence Limits		X^2 (dl)	Probability
			Lower ($\mu g.\mu l^{-1}$)	Upper($\mu g.\mu l^{-1}$)		
Macrophages	Phagocytic Index	8091	4732	22878	1.28 (3)	0.73
	NO Production	308,63	222.28	419.23	0.06 (1)	0.80
	Lysosomal Enzymes Activity	284	7564	1864176	0.72 (3)	0.86
	Myeloperoxidase Activity	3423	1307	$4.53x10^{14}$	13.86 (3)	0.003
Lymphocytes	Proliferation PHA-dependent	217.50	-	-	6.69 (1)	0.009
	Proliferation LPS-dependent	1018	570.76	2776	7.49 (3)	0.05

Combined action of *B. dalzeilii* and *P. kotschyi*: *B. dalzeilii* and *P. kotschyi* was used as mixtures in volume ratios of 3:1, 1:1 and 1:3 and the combined EC_{50} was estimated at 15411, 9728 and 589357356 $\mu g.\mu l^{-1}$ respectively in stimulation of phagocytic index. In the same ratios, the combined EC_{50} was assessed as equal to 131.9, 226.4 and 18.8 $\mu g.\mu l^{-1}$ for stimulation of lysosomal enzymes activity and 4933, 893.79 and 959.43 $\mu g.\mu l^{-1}$ for stimulation of MPO-dependent activity. In stimulation of NO production, the combined EC_{50} was estimated as evaluated as 1328 and 561.32 $\mu g.\mu l^{-1}$ respectively for ratios of 1:1 and 1:3. With the ratio of 3:1, the activity of the mixture was total in the little concentration and the EC_{50} was not assessed in the limits of the used concentrations. The combined

EC_{50} was estimated at 9830, 6.07and 640.91 µg.µl^{-1} respectively for the mixtures in volume ratios of 3:1, 1:1 and 1:3 in stimulation of lymphocytes proliferation in response to LPS. In this study, the effect of the mixtures of *B. dalzeilii* and *P. kotschyi* was important or above 50% for the different ratios and the combined EC_{50} were not calculated (**Table 3**).

TABLE 3: EC_{50}, 95% CONFIDENCE LIMITS AND PROBABILITY OF AQUEOUS EXTRACT OF *B. DALZEILII* AND *P. KOTSCHYI* IN MASS RATIOS TO SOME IMMUNOLOGICAL ACTIVITY AFTER 48H OF INCUBATION

Immunological Activity	Ratio (*B. dalzeilii/P. kotschyi*)	EC_{50} (µg.µl^{-1})	95% Confidence Limits		X^2 (dl)	Probability
			Lower (µg.µl^{-1})	Upper(µg.µl^{-1})		
Phagocytic Index	3:1	15411	6293	121548	2.38 (3)	0.50
	1:1	9728	4586	48928	0.62 (3)	0.88
	1:3	589357356	-	-	0.33 (3)	0.95
NO Production	3:1	-	-	-	-	-
	1:1	1328	750.51	10447	0.64 (1)	0.42
	1:3	561.32	-	-	75.92 (3)	0.0001
Lysosomal Enzymes Activity	3:1	131.9	69.78	179.12	0.90 (1)	0.34
	1:1	226.4	180.23	268.54	1.66 (1)	0.19
	1:3	18.8	-	-	-	-
Myeloperoxidase Activity	3:1	4933	1775	771284206	8.30 (3)	0.04
	1:1	893.79	-	-	30.66 (3)	0.0001
	1:3	959.43	401.05	7958	17.81 (3)	0.0005
Proliferation PHA-dependent	3:1	-	-	-	-	-
	1:1	-	-	-	-	-
	1:3	-	-	-	-	-
Proliferation LPS-dependent	3:1	9830	2300	$1.53x10^{17}$	2.95 (3)	0.39
	1:1	6,07	-	-	37.22 (3)	0.0001
	1:3	640,91	221.97	1927	9.25 (3)	0.026

Synergistic effect of *B. dalzeilii* and *P. kotschyi*: The volume mixtures of *B. dalzeilii* and *P. kotschyi* increased sometimes the activities of macrophages and lymphocytes proliferation than when they were used alone. The combined EC_{50} values have been segregated as ratio and the synergistic coefficient values were calculated and are presented in **Table 4**. It was observed that in some cases *B. dalzeilii* and *P. kotschyi* acted as synergist to some biological effect of macrophages and lymphocytes proliferation having synergistic coefficient values greater than 1.

TABLE 4: SYNERGISTIC COEFFICIENT OF AQUEOUS EXTRACT OF *B. DALZEILII* AND *P. KOTSCHYI* APPLIED IN DIFFERENT MASS RATIOS AFTER 48H OF INCUBATION

Immunological Activity	Ratio (*B. dalzeilii* :*P. kotschyi*)	Combined EC_{50} (µg.µl^{-1})	Synergistic Coefficient
Phagocytic Index	3:1	15411	0,09
	1:1	9728	0,21
	1:3	589357356	$5,56x10^{-5}$
NO Production	3:1	-	-
	1:1	1328	0.34
	1:3	561.3	0.65
Lysosomal Enzymes Activity	3:1	131.9	0.44
	1:1	226.4	0.26
	1:3	18.8	3.14
Myeloperoxidase Activity	3:1	4933	2.27
	1:1	893.79	7.13
	1:3	959.43	4.64
Proliferation PHA-dependent	3:1	-	-
	1:1	-	-
	1:3	-	-
Proliferation LPS-dependent	3:1	9830	0,04
	1:1	6,07	91,83
	1:3	640,91	1,12

The results shows that *B. dalzeilii* and *P. kotschyi* acted as synergist in stimulation of MPO-dependent. They were best synergized at 1:1 ratio having the highest synergistic coefficient value (7.13) followed 1:3 (4.64) and 3:1 (2.27). *B. dalzeilii* and *P. kotschyi* acted as synergist in stimulation of lysosomal enzymes activity at 1:3 ratio with synergistic coefficient value of 3.14. *B. dalzeilii* and *P. kotschyi* were also proved to act as synergist in stimulation of lymphocytes proliferation in response to LPS stimulation. In stimulation of lymphocyte proliferation induced by PHA the mixtures proved high activity more than 50% in the concentration from 160 to 2560 $\mu g.\mu l^{-1}$ used in the study. In other activity as phagocytic index and NO production, the ratios proved antagonistic having the same synergistic coefficient value less than 1.

DISCUSSION: The EC_{50} and the probability of *B. dalzeilii* and *P. kotschyi* on immunological activity related to the defense against microbes were assessed used the activity of the different concentrations ranged from 160 to 2560 $\mu g.\mu l^{-1}$. The probabilities found the extract of *B. dalzeilii* were less than 0.5 for all the studied activities while with extract of *P. kotschyi* the probabilities were less than 0.5 for MPO-dependent activity and lymphocytes proliferation. For the mixtures the probabilities were less than 0.5 excepting in stimulation phagocytic index. The less probabilities than 0.5 suggest a significant effect of the extracts and the mixtures.

The present results are in-agreement with those of several auteurs that reported the interactions of the extract compounds in modulation of the immune response [11]. From the present experiments it is very much clear that *B. dalzeilii* and *P. kotschyi* have some compounds that interact in modulation of immune response. Some of compounds present in those extracts as synergist enhance the MPO-dependent and lysosomal enzymes activity in macrophages and they could be used to control infectious diseases in this manner [12]. Certain compounds as synergist also enhance the lymphocytes proliferation in response to PHA and LPS. The augmentation of CMI to PHA and LPS as observed it is an evidence of enhanced responsiveness of T and B lymphocyte subset [13]. The reason for the enhanced MPO-dependent and lysosomal enzymes activity with combined *B. dalzeilii* and *P. kotschyi* were not examined in the experiments, but a possible explanation is that the extracts increased the enzymes activities.

In other activity, the combined *B. dalzeilii* and *P. kotschyi* showed an antagonistic effect reducing the phagocytic index and NO production. The probability explanation of this contrary interaction could be the presence of various compounds in the extracts. In other, the present results showed that the mixtures of *B. dalzeilii* and *P. kotschyi* could be used to reduce exaggerated response or to increase a low immune response [14].

CONCLUSION: It may be concluded that *B. dalzeilii* and *P. kotschyi* differ in their intensity of synergism followed the biological activity. Their synergistic efficiency thus confirmed, *B. dalzeilii* and *P. kotschyi* may be recommended for use in immune response control strategies to increase and reduce immune response. Further research is needed to understand the biological background of synergistic and antagonistic effects of the extract compounds, probably for each biological activity.

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