Evaluation of the Dietetic and Therapeutic Potential of a High Molecular Weight Hydroxycinnamate-Derived Polymer from *Symphytum asperum* Lepech. Regarding Its Antioxidant, Antilipoperoxidant, Antiinflammatory, and Cytotoxic Properties

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A water-soluble hydroxycinnamate-derived polymer (>1000 kDa) from *Symphytum asperum* Lepech. (Boraginaceae) strongly reduced the diphenylpicrylhydrazyl radical ($IC_{50} \approx 0.7$ μg/mL) and inhibited the nonenzymatic lipid peroxidation of bovine brain extracts ($IC_{50} \approx 10$ ng). This polymer exhibited only a low hydroxyl radical scavenging effect in the $Fe^{3+}-EDTA-H_2O_2$ deoxyribose system ($IC_{50} > 100$ μg/mL) but strongly decreased superoxide anion generation in either the reaction of phenazine methosulfate with NADH and molecular oxygen ($IC_{50} \approx 13.4$ μg/mL) or in rat PMA-activated leukocytes ($IC_{50} \approx 5$ μg/mL). The ability to inhibit both degranulation of azurophil granules and superoxide generation in primed leukocytes indicates that the NADPH oxidase responsible for this later effect is inhibited, pointing to the *Symphytum asperum* polymer as a potent antiinflammatory and vasoprotective agent. At all concentrations tested (0−200 μg/mL), we observed no cytotoxicity on normal human fibroblasts and neither antiproliferative effects nor proliferation activation on neoplastic cells.

Keywords: *Symphytum asperum; hydroxycinnamates; DPPH assay; lipid peroxidation; hydroxyl radical generation; superoxide anion generation; degranulation; neutrophils; growth-inhibition; growth-proliferation; human fibroblast; colon adenocarcinoma; breast adenocarcinoma*

INTRODUCTION

The generation of reactive oxygen species (ROS) beyond the antioxidant capacity of a biological system gives rise to oxidative stress (*1*, *2*), which has been implicated in the pathogenesis of a variety of human diseases (*3*) such as inflammation, cancer, atherosclerosis, diabetes mellitus, as well in the aging process (*4*, *5*). To limit the negative effects of stimuli that modify (remove) the equilibrium between the proxidant and antioxidant state in favor of the proxidant state, antioxidants able to scavenge and prevent the formation of free radicals are needed. There is compelling evidence that polyphenolic compounds might affect the formation and scavenging of ROS and influence processes involving free radical-mediated injury. Moreover, different trials provide consistent data suggesting their interest as dietary supplements in the prevention of cardiovascular disease as well as in cancer (*6*−*8*).

Previous work (*9*) showed that a crude glucofructan preparation isolated from the roots of *Symphytum asperum* Lepech. (Boraginaceae) displayed strong anticomplementary activity and inhibited the production of luminol-dependent chemiluminescence generated by zymosan-stimulated human polymorphonuclear leukocytes (PMNs). NMR studies (*10*) showed that the main polymer present in this fraction is a new water-soluble dihydroxycinnamate-derived polymer with a polyoxy-1-carboxy-2-(3,4-dihydroxyphenyl) ethylene chain (Figure 1). In the present study, we evaluated in vitro the ability of a standardized preparation of this polymer to reduce the diphenylpicrylhydrazyl radical, to inhibit lipid peroxidation, to scavenge and/or limit the generation of highly reactive superoxide anion and hydroxyl radical, and to interact with some functional responses of PMA-activated leukocytes. In addition, we also evaluated the cellular toxicity of the standardized extract by measuring the ability to induce cell growth inhibition in normal human fibroblasts and in two tumor cell lines.

MATERIAL AND METHODS

Biologicals and Chemicals. Breast cancer adenocarcinoma MCF 7 and colon adenocarcinoma DLD-1 human cell-lines were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, United-Kingdom). Normal human fibroblasts were purchased from Biopredic International (Rennes, France). This frozen culture was obtained from a 36-year-old femal abdominal surgical waste, and the cells used in this work were from the 7th to the 12th passage. Minimum essential medium with Earle's salts and Glutamax, solution of vitamins, sodium pyruvate, nonessential amino acids, PBS, and gentamicin base were purchased from Gibco-BRL (Paisley, Scotland). Fetal calf serum was obtained from Bio West (Nuaillé, France), and Nunclon 96-well microplates were from Nunc (Roskilde, Denmark).

Sterile NaCl 0.15 M was from Eurobio (Les Ulis, France); bovine brain phospholipids, ascorbic acid, EDTA, ADP, BHT, DPPH, DMSO, β-NADPH, β- NADH, deoxyribose, Hoechst 33342, NBT, PMS, and PMA were from Sigma Aldrich (L'Isle

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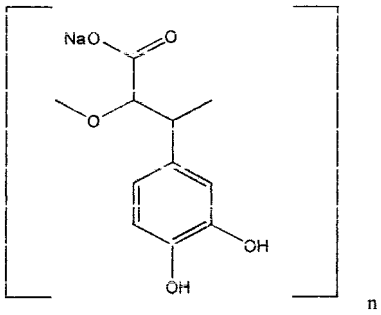

Figure 1. Structure of dihydroxycinnamate-derived polymer from *Symphytum asperum* Lepech.

d'Abeau Chesnes, France). Salts were from Merck (Darmstadt, Germany). Other chemicals were from Fluka (Buchs, Switzerlands).

Plant Extract. *Symphytum asperum* samples were collected in Georgia in June. A voucher sample was deposited in the herbarium of the Institute of Pharmacochemistry (Tbilissi). Extraction and purification were done as previously described (*10*), i.e., extraction of a crude polysaccharide extract (10.2% of dry roots), ultrafiltration through membranes with cutoff values of 1000 kDa, followed by fractional dissolution in veronal-saline buffer pH 7.35 and gel-filtration on sepharose 2B to obtain a water-soluble high-molecular weight polymer preparation with low carbohydrate (25.7%) and minor protein (1.07%) content, and three absorption bands in a UV spectrum at 252 nm (moderate), 282 nm (strong), and 286 nm (strong). The yield of phenolic polymers is 2.75% based on dry roots (*9*).

DPPH Assay. The DPPH test was carried out as described by Cuendet et al. (*11*). Briefly, 50 μL of aqueous dilutions of polymer (0−25 μg of dry product) was mixed with 5 mL of a 0.004% methanol solution of DPPH. After an incubation period of 30 min, the absorbance of samples was read at 517 nm using a Beckman DU 40 spectrophotometer. BHT, quercetin, and ascorbic acid were used as reference products.

Inhibition of Lipid Peroxidation in Liposomes. Experiments were carried out as described by Houghton et al. (*12*) with the modifications proposed by Burits and Bucar (*13*). Phospholipid liposomes were prepared from Type VII Folch bovine extract by mixing with KH_2PO_4−K_2HPO_4 buffer (5 mg/mL) and storing at 4 °C for 2 days. Before the test, the sample was sonicated under cooling with ice until a milky solution was obtained. The reaction mixture contained 500 μL of this suspension and 500 μL of solution containing the *S. asperum* polymer (from 5 ng/mL to 10 μg/mL), or an equivalent volume of distilled water for the controls, 10 mM phosphate buffer (pH 7.4), 1 mM Fe^{3+}, and 100 μL of ascorbic acid to start peroxidation. Samples were incubated at 37 °C for 1 h, and then liposome lipid peroxidation was measured by adding 1.0 mL of TBA (1% in 50 mM NaOH), 1.0 mL of TCA (2.8%), and 100 μL of BHT (2% in ethanol) in test tubes and heating at 100 °C for 20 min. After the sample was cooled, 2.5 mL of *n*-butanol was added, and the reaction mixtures were centrifugated at 3500 rpm for 5 min. The absorbance of the organic layer was measured at 532 nm. Quercetine was used as a positive control. All reagents were freshly prepared.

Inhibition of Hydroxyl Radical Formation. Assays were conducted in the Fe^{3+}−EDTA−H_2O_2 deoxyribose system using reaction mixtures containing the *S. asperum* polymer (25, 50, 75, or 100 μgl/mL, respectively), or an equivalent volume of distilled water for the controls, 100 μM ascorbic acid, 100 μM $FeCl_3$, 104 μM EDTA, 1 mM H_2O_2, 2.8 μM deoxyribose, and 20 mM phosphate buffer (pH 7.4). Hydroxyl radicals were generated by incubating the mixture at 37 °C for 60 min (*14*). The iron salt ($FeCL_3$) was mixed with EDTA before addition to the reaction mixture. The extent of desoxyribose degradation by the formed hydroxyl radicals was measured directly in the aqueous phase by the TBA test (*15*). Mannitol was assayed as a standard.

Inhibition of Superoxide Anion Generation was assayed according to the reaction of PMS in the presence of NADH and molecular oxygen. A reaction mixture containing the *S. asperum* polymer (12.5, 25, 37.5, 50, 75, or 100 μg/mL, respectively), or an equivalent volume of distilled water for the controls, was mixed with 90 μM NADH, 2.7 μM PMS, 43 μM NBT, and 19 mM phosphate buffer (pH 7.4). NBT reduction was followed by measuring absorbance of formazan at 560 nm at ambient temperature (20 °C) for 2 min. After the subtraction of the respective blanks, the reaction rate was used to calculate the percentage of inhibition relative to the addition of the *S. asperum* polymer. Mammalian SOD (from bovine erythrocytes) was used as the reference product.

Inhibition of Some Functional Responses of Rat PMA-Activated Leukocytes. *Elicitation of Rat Leukocytes.* Peritoneal leukocytes were elicited from male Wistar rats (150−200 g) and suspended in HBSS as described by Godoy et al. (*16*). Cell viability based on trypan blue was greater than 95% (2.5×10^6 cells).

Inhibition of Superoxide Generation. Assays were set up in triplicate with peritoneal leukocytes preincubated at 37 °C for 10 min in HBSS before stimulation with PMA (1 μM). Superoxide generation was estimated as the reduction of ferrichrome C at 550 nm (*17*). SOD (37.5 U/mL) was used as a standard.

Inhibition of Lactate Dehydrogenase (LDH) and β-Glucuronidase Release from Rat Peritoneal Leukocytes. Assays were conducted in supernatants from leukocytes suspensions (1.25×10^6 cells in HBSS) stimulated with the calcium ionophore A23187 (final concentration 1 μM) as described by Godoy et al. (*16*). SOD (37.5 U/mL) was used as a standard.

In Vitro Evaluation of Cytotoxic and/or Growth-Proliferation Effects on Normal Human Fibroblasts and Human Tumor Cell Lines. *Cell Culture.* Stock cell cultures were maintained as monolayers in 75-cm^2 culture flasks in Glutamax Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal calf serum, 5 mL of a 100× solution of vitamins, 5 mL of 100 mM sodium pyruvate, 5 mL of 100× nonessential amino acids, and 2 mg of gentamicin base. Cells were grown in a humidified 37 °C incubator containing 5% CO_2.

Survival Assays. Cells were plated at a density of 5×10^3 cells/well in 96-well microplates containing 190 μL of culture medium/well and allowed to adhere for 16 h before treatment. Stock solution (2 mg/mL) of extract was prepared in sterile NaCl 0.15 M and kept at −20 °C until use. Then, 10 μL of a 10× extract solution in NaCl 0.15 M was added. A continuous drug exposure protocol for 48 h was used, and the maximal concentration tested was 200 μg/mL. A positive control (DMSO 2.5%, v/v) was tested on each plate.

The antiproliferative and growth-stimulation effects were assessed using Hoechst 33342 assay to determine the DNA cellular content after cell lysis.

Hoechst 33342 Assay. The Hoechst 33342 assay was carried out according to Rago et al. (*18*) with minor modifications. Before use, plates were rinsed with 200 μL of PBS at 37 °C using an automatic microplate washer (Cell Wash, Labsystems, Helsinki, Finland) and then were placed at −80 °C until the Hoechst assay. On the day of the assay, plates were thawed at room temperature for 10 min, then 100 μL of a 0.01% (m/v) SDS solution in sterile distillated water were distributed in each well, and plates were incubated 1 h at room temperature and then frozen again at −80 °C for 1 h. After thawing (approximatively 15 min) of the sample, 100 μL of Hoechst 33342 solution (30 μg/mL in an hypersaline buffer containing 10 mM Tris HCl, pH 7.4, 1 mM EDTA, and 2 M NaCl) were added to each well, and plates were incubated for 1 h in the dark at room temperature. Fluorescence was measured at 360/460 nm on a microplate fluorescence reader (Fluoroskan Ascent FL, Labsystems). Using these conditions, fluorescence in experimental wells is proportional to the number of adherent cells, and the DNA content is calculated as a percentage of that in control wells (containing solvent) after subtraction of blank values. Each experiment was performed twice.

Table 1. Evaluation of Antioxidant Activity Using DPPH Test[a]

test compound	IC_{50} (μg/mL)
S. asperum polymer	0.72
references	
quercetine	1.33
BHT	12.20
ascorbic acid	3.78

[a] Values are expressed as mean for 3 tests.

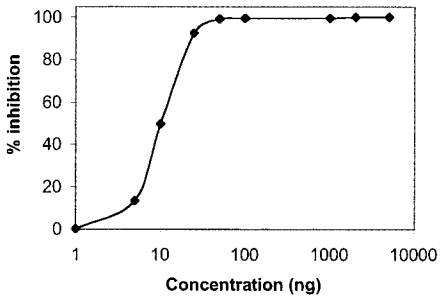

Figure 2. Inhibition of lipid peroxidation with *S. asperum* extract. (Values are expressed as mean for 3 tests).

Statistics. Statistical analysis was performed using Student's *t*-test. The inhibitory concentration 50% (IC_{50}) was calculated by regression analysis of the concentration−response data.

RESULTS

DPPH Assay. Results in Table 1 demonstrated the strong ability of the *S. asperum* polymer to act as a donor for hydrogen atoms or electrons. The reduction of the stable radical DPPH to the yellow colored diphenylpicrylhydrazine was obtained with an IC_{50} of 0.72 μg/mL instead of 1.33, 12.20, and 3.78 for quercetine, BHT, and ascorbic acid, respectively.

Antilipoperoxidant Effect. The *S. asperum* polymer exhibits a strong dose-dependent antilipoperoxidant effect in phospholipids liposomes (Figure 2). The latter undergo rapid nonenzymatic peroxidation when incubated with Fe^{3+} in the presence of a reducing agent. The extent of lipid peroxidation was classically assayed by measuring the amount of pink pigment resulting from the reaction of 2-thiobarbituric acid (and oxidation products) on aldehydes including malondialdehyde (*19*). Under the conditions used, 10 ng of the *S. asperum* polymer were able to inhibit 50% of lipid damage, whereas the IC_{50} of quercetine is 2 μg/mL, showing that the antiperoxidant activity of the *S. asperum* polymer is about 200 times higher than that of the reference material (Figure 2).

Hydroxyl Radical and Superoxide Anion Scavenging Effect. The radical-scavenging effect of the *S. asperum* polymer varied with the radical tested. The ability to scavenge and prevent the formation of hydroxyl radical was low and only detectable at concentrations higher than 50 μg/mL. The IC_{50} was higher than 100 μg/mL and was not reached. In contrast, the polymer showed good potency as a superoxide scavenger (IC_{50}: 13.4 μg/mL). In the present assay, superoxide anion generation is based on the interactions between NADH, PMS, molecular oxygen, and NBT. The inhibition obtained with 37.5 μg of polymer was similar to that observed with 12.1 bovine SOD units.

Interaction with Some Functional Responses of PMA-Activated PMNs. Primed PMNs were chosen because they are specialized in releasing large amounts of H_2O_2 and superoxide (oxidative burst) to kill invading microorganisms and have fundamental roles in allergy and inflammation (*20*). The polymer was tested for its potent ability to inhibit some functional responses on rat PMA-activated leukocytes. Results (Table 2) demonstrated a strong, dose-dependent inhibition of (i) superoxide anion generation and (ii) leukocytes azurophil granular secretion (monitored by β-glucuronidase release) and no alteration of cellular integrity (assayed as LDH response).

Table 2. Inhibition of Hydroxyl Formation and Superoxide Generation

product	final conc (units/mL)	inhibition of hydroxyl radical formation (%) $IC_{50} > 100$ μg/mL	inhibition of superoxide anion generation (%) $IC_{50} \approx 13.4$ μg/mL
		S. asperum Polymer	
	12.5 μg		47.546 ± 1.376[b]
	25 μg	10.393 ± 1.143	62.172 ± 1.471[b]
	37.5 μg		85.239 ± 0.196[b]
	50 μg	13.047 ± 0.967[b]	84.367 ± 0.123[b]
	75 μg	16.564 ± 1.045[b]	85.183 ± 0.563[b]
	100 μg	19.273 ± 1.023[b]	85.725 ± 0.259[b]
		References	
mannitol	10^{-3} M	50.931 ± 0.767[b]	
bovine SOD	12.1 U		85.614 ± 0.234[b]

[a] Values are expressed as mean ± SEM for 6 tests. [b] Significant ($p < 0.01$) differences from control group.

Table 3. Interaction of *S. asperum* Polymer with Superoxide Anion and Neutrophils Functions[a]

control	inhibition of LDH release (% vs control)	inhibition of β-glucuronidase release (% vs control)	inhibition of superoxide generation (% vs control)
		S. asperum Polymer	
5 μg/mL	0.08 ± 0.03		53.92 ± 0.03[a]
10 μg/mL	0.05 ± 0.03	29.49 ± 0.17[a]	83.46 ± 0.06[a]
25 μg/mL	0.04 ± 0.02	41.43 ± 0.23[a]	91.08 ± 0.15[a]
50 μg/mL	0.06 ± 0.04	57.67 ± 0.32[a]	92.15 ± 0.12[a]
		References	
bovine SOD (37.5 U/mL)			100 ± 0.01

[a] Values are expressed as mean ± SEM for 6 tests. [b] Significant ($p < 0.01$) differences from control group.

In Vitro Evaluation of Growth-Inhibition and/or Growth-Proliferation Effects on Normal Human Fibroblasts and Human Tumor Cell Lines. Colon adenocarcinoma DLD-1 and breast adenocarcinoma MCF 7 were chosen for evaluating antineoplastic potential. Evaluation was done after 48 h of continuous contact with the polymer (0−200 μM/mL), by determination of cellular DNA content with Hoechst 33342. Since under the conditions used, DNA content is proportional to cell biomass, the method allows the evaluation of both cytotoxicity and proliferating effects. Concentrations higher than 200 μg/mL were not assayed because the *S. asperum* polymer was not entirely soluble in the culture medium at concentrations up to 200 μg/mL. No antiproliferative effects were observed in neoplastic cells or in normal fibroblasts, and no growth stimulation was found.

DISCUSSION

Highly reactive superoxide and hydroxyl radicals are continuously produced in cells (as byproducts of oxidative phosphorylation in mitochondria and during fatty acid oxidation in peroxisomes) but are usually quickly removed by efficient defense systems. Background levels

can transiently increase in response to different stimuli such as chemical or physical stress, biological threats (infections or antigen response), or hormonal signals (for instance, IL-1, TNF, leukotriens). Abnormal production of ROS is also observed in numerous human pathologies (*21*). When the defense system is overwhelmed, ROS escaping destruction may induce deleterious effects as DNA strand breaks, lipid peroxidation, and oxidative damage of proteins. To explore ways to limit oxidative damage, researchers have studied scavengers that directly (or indirectly) react with ROS and thereby neutralize their reactivity (*21*).

The preparation tested appears to have a high antioxidative potential, without cytotoxicity in PMNs or in fibroblasts and without any growth stimulatory effects in cancer cells in contrast with fractions from *Symphytum officinale* (*22*). In addition, it exhibits in vitro a strong dose-dependent antilipoperoxidant activity and inhibits the generation of superoxide anion, a highly reactive ROS intermediate produced by phagocytic cells during the oxidative burst and by other cell types (including lymphocytes, fibroblasts, and vascular endothelial cells in response to different stimuli) (*3*). The ability to inhibit both degranulation of azurophilic granules and superoxide generation in PMA-activated leukocytes indicates that the NADPH oxidase responsible for this process is inhibited. It is known that (i) the stimulation of NADPH oxidases in the plasma membrane induces a transient increase of ROS which mediates components of the inflammatory response, with production of migratory factors, as cyclic nucleotides and eicosanoids, and that (ii) superoxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of PMNs to the endothelium, and stimulation of platelet aggregation allowing a self-propagating response (*21*). The ability to inhibit NADPH oxidase points to the *S. asperum* dihydroxycinnamate-derived polymer as a potent antiinflammatory and vasoprotective agent. Aqueous extracts from *Symphytum* species are used in traditional medicine as antiinflammatory and wound-healing agents. Their antiinflammatory activity is usually related to the presence of triterpenoid saponins, which have been reported to inhibit prostaglandin biosynthesis and PAF-induced exocytosis (*23*). Our results demonstrated that the water-soluble dihydroxycinnamate-derived polymer from the *S. asperum* (2.75% of dry roots) may participate in the global activity of the plant extract. In addition, because of its strong antilipoperoxidant activity, it may partly prevent both LDL oxidation and formation of byproducts resulting from lipid lipoperoxidation, and therefore, it may have a beneficial effect in the prevention of atherosclerosis and cardiovascular diseases. It might also have a beneficial effect for patients suffering from predisposing inflammatory conditions such as ulcerative colitis and Crohn's disease. Previous reports have shown that (i) the colonic mucosa of patients suffering from such diseases or cancer generates higher amounts of ROS than normal tissue and (ii) antioxidant phenolic compounds (such those present in olive oil) are potent inhibitors of ROS generation by the fecal matrix (*24*). New assays are needed to evaluate the activity of the standardized extract in these different indications.

Finally, we evaluated the antiproliferative potential of the *S. asperum* polymer on some neoplastic cells. There is growing evidence that oxidizing agents generated during lipid peroxidation (initiated and propagated by ROS) lead to the formation of highly promutagenic DNA adducts (*24*, *25*) and that ROS are involved in the etiology of "fat-related neoplasms" such as cancer of the breast and colorectum (*24*). On the basis of its high antioxidant and antilipoperoxidant activity, the *S. asperum* polymer might have chemopreventive potential. Recent reports showed that different antioxidant agents that show antimutagenic and anticarcinogenenic activities in vivo exhibit, in vitro, antiproliferative effect specifically on cancer cells (*26*−*32*). The exact mechanisms involved are not yet known; however, specific interaction with steroid receptors (*27*), prevention of protein kinase C translocation and activation (*31*), decrease of ornithine decarboxylase (ODC) activity (*26*), and/or ability to act as a pro-oxidant have been reported (*28*). Assays were done on colon adenocarcinoma DLD-1 and breast adenocarcinoma MCF 7 cell-lines. MCF 7, an hormone sensitive cell-line, was chosen because previous data reported that some polyphenols decrease cell proliferation of hormone-sensitive cell lines in a dose- and time-dependent manner (*27*) via specific interactions with steroid receptors. On the basis of the chemical structure of the *S. asperum* polymer, we expected an antiproliferative effect. However, at the higher concentration investigated (200 μg/mL), neither growth inhibitory effects nor proliferation activation was observed. At least two hypotheses may be assumed: (i) due to its high molecular weight (>1000), the polymer is not able to cross the cellular membrane and/or to act on intracellular target(s) or (ii) its poor scavenging ability on hydroxyl radicals is involved in its inefficacy. Indeed, to date, the paradoxical cytotoxic effect of the antioxidant on cancer cells is not explained, and a role of the hydroxyl radical could not be determined. It is obvious that further experiments are needed to clarify these points.

To conclude, our results show that the dihydroxycinnamate-derived polymer from the *S. asperum* presents dietetical and/or therapeutical potential and that the activity/safety index of the preparation tested justifies new pharmacological evaluations. Further research is needed to evaluate in vitro and in vivo the ability of this polymer (i) to block NF-kB activation, a transcriptional factor, which plays a crucial role in acute phase inflammatory, immune, and cancer responses and (ii) to prevent mutagenesis and cellular transformation into malignant neoplastic form.

ABBREVIATIONS USED

Adenosine-5′-diphosphate potassium (ADP); butylhydroxytoluene (BHT); diphenylpicrylhydrazyl radical (DPPH); dimethyl sulfoxide (DMSO); ethylenediaminetetra acetic acid (EDTA); Hank's balanced salt solution (HBSS); inhibitory concentration 50% (IC_{50}); interleukin-1 (IL-1); lactate deshydrogenase (LDH); low-density lipoprotein (LDL); β-dihydronicotinamide-adenine dinucleotide phosphate (β-NADPH); β-dihydronicotinamide-adenine dinucleotide (β-NADH); nitroblue tetrazolium (NBT); phenazine methosulphate (PMS); phorbol myristate acetate (PMA); phosphate buffer saline (PBS); platelet activating factor (PAF), polymorphonuclear leukocytes (PMNs); sodium dodecyl sulfate (SDS); superoxide dismutase (SOD); reactive oxygen species (ROS); thiobarbituric acid (TBA); trichloracetic acid (TCA); tumor necrosis factor (TNF).

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