Release of Intermediate Reactive Hydrogen Peroxide by Macrophage Cells Activated by Natural Products

Raquel Regina Duarte MOREIRA,*,[a] Iracilda Zeppone CARLOS,[b] and Wagner VILEGAS[c]

Departamento de Princípios Ativos Naturais e Toxicologia Brasil,[a] Departamento de Análises Clínicas,[b] Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista "Júlio de Mesquita Filho," CP 502, CEP 14801–902, Araraquara, São Paulo, Brasil, Departamento de Química Orgânica,[c] Instituto de Química, Universidade Estadual Paulista, Araraquara, São Paulo, Brasil. Received June 2, 2000; accepted October 3, 2000

By determining the hydrogen peroxide (H_2O_2) released in cultures of peritoneal macrophage cells from Swiss mice, we evaluated the action of 27 vegetable compounds (pristimerin, tingenone, jatrophone, palustric acid, lupeol, cladrastin, ocoteine, boldine, tomatine, yohimbine, reserpine, escopoletin, esculine, plumericin, diosgenin, deoxyschizandrin, *p*-arbutin, mangiferin, and others) using a 2 mg/ml solution of each compound (100 μg/well). Macrophages are cells responsible for the development of the immunological response reaction, liberating more than one hundred compounds into the extracellular environment. Among these are the various cytokines and the intermediate compounds of nitrogen (NO) and oxygen (H_2O_2). This coordinated sequence of biochemical reactions is known as the "oxidative burst." When we compared the results with those obtained with zymosan (an important stimulator of H_2O_2) we observed that the compounds showing the highest activity were substances 2 (tingenone), 16 (reserpine) and 20. Other substances such as compounds 1, 4, 5, 6, 8, 12, 13, 14, 15, 17, 19, 23, 24, 26, and 27 also showed a certain activity, but with less intensity than the aforementioned ones. Compounds 3, 7, 9, 10, 11, 18, 21, 22 and 25 presented no activity. These results suggest that natural products (mainly tingenone and reserpine and others) with different chemical structures are strong immunological modulators. However, further tests are needed to determine the 'oxidative burst' in future studies.

Key words natural product; macrophage; hydrogen peroxide

Plants are important sources of biologically active natural products which differ widely in terms of structure and biological properties. Some of the isolated plant constituents such as flavonoids, tannins, alkaloids, terpenes and others are responsible for many biological activities, such as analgesic activity, antiinflammatory activity, antiviral activity, antispasmodic activity, antiallergenic activity, and others.[19] We can attribute this to the molecular diversity of these products.

Many human physiological activities such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions have also been assigned to different compounds.[7]

One of the biological activities of plant compounds that has attracted great interest is the capacity to stimulate macrophages. Macrophages are known to play an important role in host defense mechanisms. Being the first cells to participate in the immunological response, macrophages can be activated by a variety of stimuli and their principal functions include the phagocytosis of foreign particles, showing the presence of antigens, and the production of cytokines and of the intermediate compounds of oxygen (H_2O_2) and nitrogen (NO).[4] Recent studies have also suggested that H_2O_2 plays an important role in the functions of macrophages.[3,11,16,17]

Currently, there is a strong tendency to study natural compounds that may be involved in the modulation of the immunological system. A variety of materials derived from plants pertaining to the different classes of active agents have been reported to stimulate the immune system, with macrophage stimulation occurring in many cases.[1,2,4,7,9—15,18]

A simple, rapid and inexpensive method to measure the hydrogen peroxide (H_2O_2) produced by cells in *in vitro* culture is based on the horseradish peroxidase (HRPO)-mediated oxidation of phenol red by H_2O_2 which results in the formation of a compound demonstrating increased absorbance at 620 nm.

The products of the oxidative burst are used to kill phagocytosed pathogens and for the extracellular destruction of other cells. In addition to H_2O_2 and O_2^-, two other highly reactive oxygen derivatives have been implicated in the killing process, *i.e.*, hydroxyl radicals ($\cdot$OH) and singlet oxygen ($^1O_2$). This coordinated sequence of biochemical reactions is initiated by an increase in oxygen uptake followed by the one-electron reduction of oxygen to superoxide anion (O_2^-) using NADPH or NADH as the electron donor and catalyzed by an NAD(P)H oxidase. O_2^- is subsequently converted to hydrogen peroxide (H_2O_2).[15,16]

While the relative importance of O_2^-, $\cdot$OH and $^1O_2$ in oxygen-dependent killing is still debated, the role of H_2O_2 in both intra- and extracellular toxicity is well-established.

The objective of the present study was to investigate the effect of 27 natural products on mouse peritoneal macrophage functions by the liberation of H_2O_2.

MATERIAL AND METHODS

Animals Six-week old male Swiss mice weighing 18 to 25 g were supplied by the Animal House of the Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista-UNESP, Araraquara-São Paulo-Brasil. The animals were maintained in a polycarbonate box, with water and food available *ad libitum*.

Plant Materials Natural products with varying chemical structures were isolated from various plants (see structures, Fig. 1). The purification procedures were performed by the Laboratório de Fitoquímica, Departamento de Química Orgânica, Universidade Estadual Paulista "Júlio de Mesquita Filho", Araraquara, São Paulo, Brasil, where they are deposited. Samples were checked for purity by TLC, GC-FID

* To whom correspondence should be addressed. e-mail: moreirar@fcfar.unesp.br
© 2001 Pharmaceutical Society of Japan

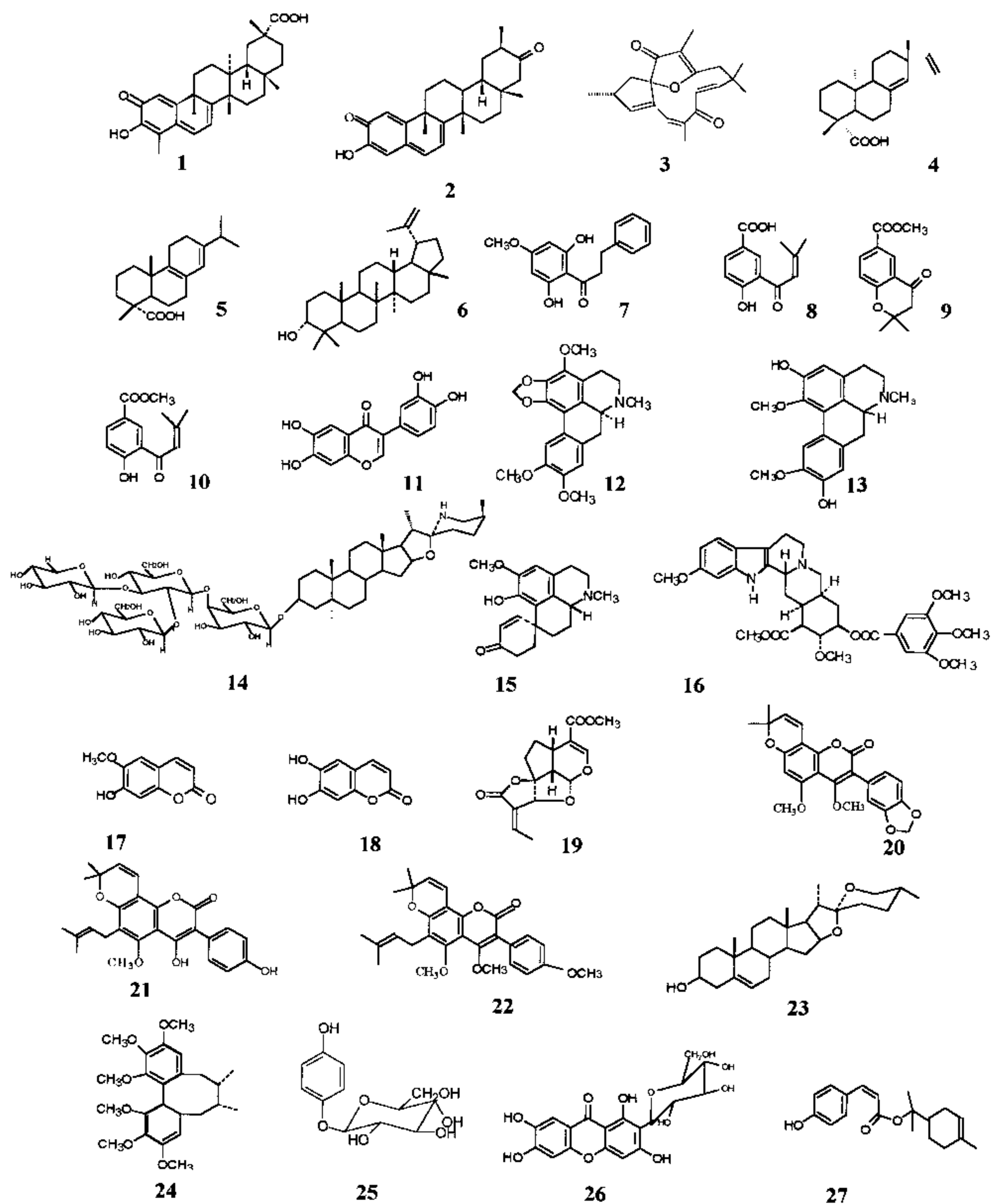

Fig. 1. Structures of Natural Products

1, pristimerin; **2**, tingenone; **3**, jatrophone; **5**, palustric acid; **6**, lupeol; **11**, cladrastin; **12**, ocoteine; **13**, boldine; **14**, tomatine; **15**, yohimbine; **16**, reserpine; **17**, escopoletin; **18**, esculine; **19**, plumericin; **23**, diosgenin; **24**, deoxyschizandrin; **25**, *p*-arbutin; **26**, mangiferin.

and/or HPLC-UV methods. Purity was 95% or higher.

Macrophage Cells and Determination of Hydrogen Peroxide (H_2O_2) Using the *in vitro* culture method and natural products, we determined the reactive oxygen compounds.[15,16] The method consists of the determination of the liberation of hydrogen peroxide (H_2O_2) in the culture of peritoneal macrophages from Swiss mice. Peritoneal thioglycolate-elicited macrophages from naive mice were obtained as reported previously.[3] The peritoneal cavity was washed with 10 ml of cold phosphate buffered solution (PBS), the resulting suspension was pelleted at 4 °C by centrifugation for 5 min at *ca.* 300 g, and the supernatant was removed. The

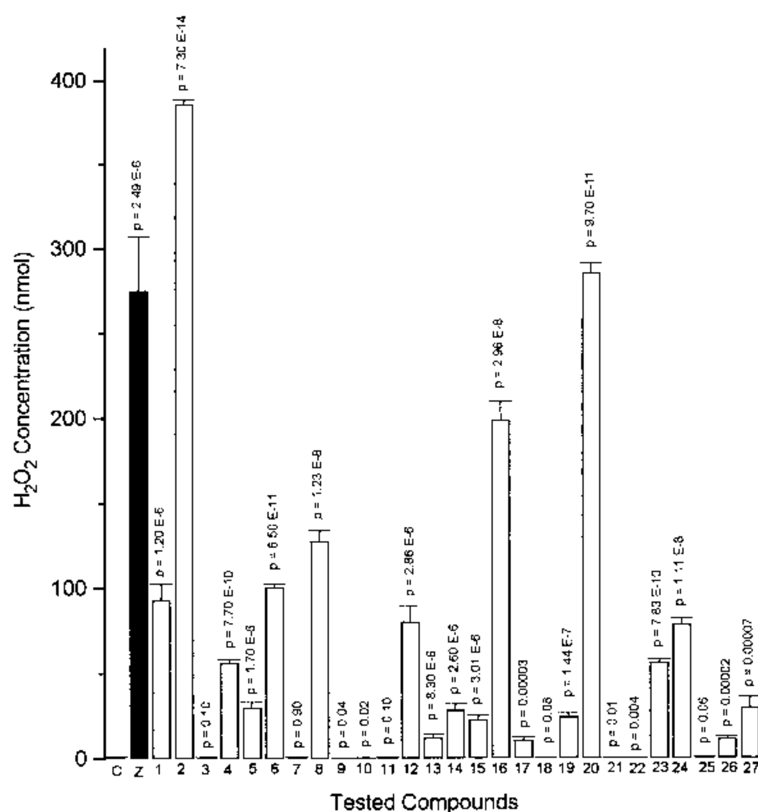

Fig. 2. Effects of Natural Products on Liberation on Hydrogen Peroxide Production by Mice Peritoneal Macrophages *in Vitro*

The macrophages were cultured for 1 h in the presence of Control DMSO (C), Zymosan (250 μg/well) (Z) and isolated compounds (100 μg/well). Each bar represents the mean±S.D. of four animals. Representative results of one experiment repeated four times are given. Significantly different from control group, $p<0.05$.

cells were resuspended at a concentration of 2×10^6 cells/ml in a solution of phenol red containing 140 mM NaCl, 10 mM potassium phosphate, pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red, and type II horseradish peroxidase, 0.01 mg/ml, (Sigma). Aliquots of 0.1 ml were transferred to flat-bottomed 96-well culture plates (Corning). A 50 μl volume of the natural products solution (2 mg/ml, Sigma) or 50 μl of dimethyl sulfoxide (DMSO) was added. We used control without cells (culture media+DMSO); control with cells (culture media+cells+DMSO); natural products dissolved in DMSO+culture media+cells and positive control (Zymosan+culture media+cells). The samples were incubated for one hour at 37 °C in a humid atmosphere and the reaction was stopped by the addition of 10 μl of 4 N NaOH. Experiments were run in quadruplicate. Absorbance was determined with an automatic ELISA photometer with a 620 nm filter. The results were expressed as nanomoles of $H_2O_2/2\times10^5$ peritoneal cells, from a standard curve established in each test consisting of known molar concentrations of H_2O_2 in buffered phenol red.

Statistical Analysis Data are expressed as mean±standard deviation, and the Student's *t*-test was used to determine the significance of the differences between the control and experimental groups.

RESULTS AND DISCUSSION

Many of our present medicines are directly or indirectly derived from higher plants. While several classic plant drugs have lost much ground to synthetic competitors, others have gained a new investigational or therapeutical status in recent years. Clinical plant-based research has made particularly rewarding progress in the important fields of therapy, such as yohimbine in male sexual dysfunction, reserpine as an antihypertensive agent, and boldine for digestive dysfunction.[19] Diterpenoids isolated of plants from the genus *Sideritis* (Lamiaceae) have been used for their antiinflamatory and gastroprotective properties.[5]

Reactive oxygen metabolites (H_2O_2) have been suggested as potentially important signaling molecules in both intra- and intercellular reactions in a number of different cell types. It has been well documented that reactive species such as hydrogen peroxide (H_2O_2) are produced by activated inflammatory cells.[18]

Figure 2 shows the concentrations of the control group DMSO, of zymosan and of 27 natural compounds that presented a stimulatory effect detected by the determination of reactive compounds of oxygen (H_2O_2).

The present study indicates that some natural products possess stronger activity than zymosan. The data presented in Fig. 2 summarize the immune response expressed as nmol after 1 h of incubation of several natural products. The compounds with a higher modulatory activity on the immune system were 2 and 20, which were able to release 385.81 and 285.16 nmol of H_2O_2, respectively, while zymosan (a powerful immunostimulator) was able to release 275.16 nmol. Compound **16** also released a significative amount of H_2O_2 (198.91 nmol) when compared to zymosan.

Comparison among compounds **8**, **9** and **10** showed a marked decrease in the immune activity when the COOH

group is methylated. On the other hand, the introduction of a COOH group in the E-ring of **1** contributes to a decrease in the strong activity of **2**.

The high activity of **20** was also strongly reduced by removing the methylenedioxide group and by introducing the isoprene unit into the A-ring, as shown in compounds **21** and **22**. Alteration in the chemical structure of the A and B rings of the aporfinic alkaloids (**12, 13**) also led to a significant decrease in their activity. The activity of **12** was about five times higher than that of **13**.

Although the activities of compounds **5**, **13**, **15**, **17**, **19**, **27** were about ten times lower than that of zymosan, they were still higher than that of the control.

Comparison between compounds **23** and **14** showed that the sugar unit, the absence of the double bond and the nitrogenated E-ring of **14** did not significantly affect their activities, since compounds **23** and **24** presented a statistically identical activity.

Though the two coumarins (**17**, **18**) did not present significant activity, in this case methylation of OH-6 led to a slightly higher activity of **17**.

Compounds **3**, **7**, **9**, **10**, **11**, **18**, **21**, **22**, and **25** did not present any activity.

This note is the first communication of the activity of these purified components on macrophage cells.

Many immunomodulating agents such polysaccharides (*e.g*., zymosan), protein-bound lipopolysaccharides (LPS) and proteins (*e.g*., concanavalin A, phytohemaglutinin) have been of interest in clinical research[6,14] which have been reported to act primarily on cellular rather than humoral immune responses to restore the immunocompetency of impaired hosts without hyperstimulating the normals. They augment macrophage chemotaxis, phagocytosis and promote interaction with other immunoregulatory lymphoid cells.[8]

On the basis of the results obtained, we may conclude that natural products might contribute to the induction of immunostimulatory effects. However, further tests involving the 'oxidative burst' (O_2^-), cytokines and NO production are needed.

Therefore their mechanistic pharmacology is likely to be complex. Further phytochemical and pharmacological investigations of these compounds is desirable too.

Acknowledgements The authors are grateful to Marisa Campos Polesi Placeres, the technician of the Laboratório de Imunologia Clínica da Faculdade de Ciéncias Farmacêuticas of UNESP, Araraquara, São Paulo, Brazil.

REFERENCES

1) Abdul K. M., Ramchender R. P., *Immunopharmacol*., **30**, 231—236 (1995).
2) Barsucov A., Vetoshkin A., Vorontov E., Dubichev A., *Planta Med*., **56**, 696— 697 (1990).
3) Carlos I. Z., Zini M. M. C., Sgarbi D. B. G., Angluster J., Alviano C. S., Silva C. L., *Mycopathologia*, **127**, 189—194 (1994).
4) Hase K., Basnet P., Kadota S., Namba T., *Planta Med*., **63**, 216—219 (1997).
5) Heras B., Hoult J. R. S., *Planta Med*., **60**, 501—506 (1994).
6) Jain D., Kaur K., Sundaravadivel B., Salunke D. M., *J. Biol. Chem*., **26**, 16098—16102 (2000).
7) Janeway C. J., "Immunobiology: The Immune System in Health and Disease," ed. by Travers P., Garland Publishing Inc., New York, 1997.
8) Kapil A., Sharma S., *J. Ethnopharmacol*., **58**, 89—95 (1997).
9) Kim H. M., Lee S. O., Lee E. H., *Immunopharmacol*., **21**, 295—306 (1999).
10) Kreher B., Neszmélyi A., Jurcic K., Wagner H., *Planta Med*., **56**, 572—573 (1990).
11) Marques M. F., Araujo A. R., Bolzani V. A. R., Young M. C. M., Placeres M. C. P., "Jornada Farmacêutica da UNESP," ANAIS (abstract 65) (1999).
12) Mathew S., Kuttan G., *Fitoterapia*, **70**, 35—43 (1999).
13) Nores M. M., Correges M. C., Benencia F., Coulombié F. C., *J. Ethnopharmacol*., **55**, 99—106 (1997).
14) Nose M., Terawaki K., Oguri K., Ogihara Y., Yoshimatsu K., Shimomura K., *Biol. Pharm. Bull*., **21**, 1110—1112 (1998).
15) Pick E., Keisari Y., *J. Immunol. Methods*, **38**, 161—170 (1980)
16) Pick E., Mizel D., *J. Immunol. Methods*, **46**, 211—226 (1981) .
17) Puri A., Saxena K. C., Srivastava V., Tandon J. S., *J. Ethnopharmacol*., **42**, 31— 37 (1994).
18) Rochele L. G., Fischer B. M., Adler K. B., *Free Radical Biology Medicine*, **24**, 863—868 (1998).
19) Simões C. M. O., Schenkel E. P., Gosamann G., Palazzo de Mello J. C., Mentz L. A., Ros Petrovick P., "Farmacognosia: Da Planta ao Medicamento," ed. by U.F.R.G.S., Porto Alegre, 1999.