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Research Article

Phytochemical Screening and Evaluation of the Antioxidant and Antiplasmodial Activities of "*Ahoutou*", a Recipe Used in the Treatment of Malaria in Ivory Coast

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Abstract

Recipes based on medicinal plants are frequently used to treat malaria in Ivory Coast. Among these recipes is Ahoutou, made from a mixture of the leaves of three plants, one of the most widely used in the village of Djahakro in the commune of Yamoussoukro. However, the use of medicinal plant-based recipes in the fight against malaria must be based on scientific evidence of safety, efficacy and quality. The aim of this study is to provide a scientific, phytochemical and pharmacological basis for the traditional therapeutic use of Ahoutou. To this end, a phytochemical study of the Ahoutou extract was carried out, the antioxidant activity was assessed using the DPPH test and then the *in vitro* anti-plasmodial activity was carried out on clinical isolates and on the reference strain Dd2. The phytochemical study revealed the presence of polyphenols, flavonoids, alkaloids, tannins, saponins, steroids and triterpenes. The antioxidant activity test showed that Ahoutou was capable of reducing the DPPH free radical. Ahoutou showed promising anti-plasmodial activity (IC50 =12.86 µg/mL) in the clinical strain and moderate activity (IC50 =23.40 µg/mL) in the reference strain. Our results would justify the use of this recipe in the treatment of malaria. We plan to study the toxicity of Ahoutou.

Keywords

Ahoutou, Antioxidant, Antiplasmodial Activity

1. Introduction

Malaria is a potentially fatal parasitic disease transmitted to humans by female mosquitoes of the genus *Anopheles*, and accompanied by various symptoms, mainly fever. Today, this disease is a public health problem in tropical and subtropical regions of the world [1]. According to the latest WHO report, the number of malaria cases worldwide

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is estimated at 228 million, with 405,000 deaths in 2018 [2]. Most cases (213 million or 93%) and deaths (94%) in 2018 occurred in Africa. Sub-Saharan Africa remains the region most affected by this disease worldwide. In fact, nineteen sub-Saharan African countries and India accounted for almost 85% of all malaria cases worldwide [2]. Côte d'Ivoire is not immune to this situation. Indeed, malaria remains a major public health problem, due to its high frequency, severity and significant socio-economic consequences. Malaria alone accounts for 33% of consultations and is the leading cause of hospitalization in health centers. Pregnant women and children under five are the most affected. The number of malaria-related cases was 3,557,891 in 2017, with a death toll of 3,222 [3]. Effective medicines are therefore needed to combat this scourge. Faced with such a public health problem, control strategies have been developed. The use of chloroquine and monotherapies was abandoned in favor of combination therapies based on artemisinin or its derivatives (ACT). Today, artemisinin-based combination therapy (ACT) is used as first-line treatment for uncomplicated *Plasmodium falciparum* malaria in over 100 countries [4]. Unfortunately, in recent years, parasite resistance to artemisinin has been detected in four countries: Cambodia, Myanmar, Thailand and Vietnam [5]. The WHO believes that if artemisinin resistance were to spread on a large scale, the consequences for the health of populations would be disastrous. In the hope of finding an appropriate response to the resistance observed in *Plasmodium falciparum*, research into medicinal plants as reservoirs of new active ingredients has been encouraged [6]. Several research projects have already been carried out in Africa in general, and in Côte d'Ivoire in particular, with a view to developing medicinal plants. However, treatment of malaria is becoming increasingly costly due to the resurgence of the disease, and treatment is not always within the reach of people in developing countries. As a result, these populations are turning to plant-based combinations from traditional health practitioners. In traditional Ivorian medicine, traditional healers offer various plants and recipes based on plant combinations to treat illnesses such as hypertension and malaria [7]. These include "Ahoutou", a local recipe from the village of "Djahakro" in the commune of Yamoussoukro (Bélier region). This recipe is widely used in the treatment of malaria in this locality, and seems to be very popular with the villagers. According to the traditional practitioner who developed "*Ahoutou"*, this recipe is made from the leaves of three (3) plants: *Azadirachta indica*, *Cymbopogon citratus* and *Psidium guajava*. To date, there are no scientific data on Ahoutou's anti-plasmodial properties. The overall aim of this study is to provide a scientific, phytochemical and pharmacological basis for the traditional therapeutic use of Ahoutou.

2. Materials and Methods

2.1. Hardware

2.1.1. Experimental Product

The experimental product studied is a local recipe from "Djahakro", a village in the commune of Yamoussoukro, developed by a tradithérapeute called Monsieur Ahoutou. The recipe is a decoction made from the leaves of *Azadirachta indica*, *Cymbopogon citratus* and *Psidium guajava*. It is a fluid, brown solution with a bitter taste.

2.1.2. Biological Material

The biological material also included the chloroquine-resistant *Plasmodium falciparum* Dd2 reference strain, and four *Plasmodium falciparum* clinical isolates from patients with uncomplicated malaria obtained at the Anonkoua-Koutéhealth center in the Abobo commune (Abidjan, Côte d'Ivoire). These include ANK137, ANK138, ANK139 and ANK140). Group O-positive blood from healthy individuals was also used.

2.2. Methods

2.2.1. Obtaining Ahoutou Dry Extract

"Ahoutou" *was* supplied in the form of a decoctate by a tradithérapeute who produces and markets it by hand. The decoctate is a brown liquid. After filtration, the decoctate was concentrated in a vacuum rotavapor at 46 °C. The extract was then oven dried at 46 °C for 24 hours. The powders obtained were weighed and then stored in a hermetically sealed sterile bottle for the various tests.

2.2.2. Phytochemical Screening

Phytochemical analyses were carried out according to the methods described by Dohou et al [8]. Alkaloids were identified by the Dragendorff reaction; flavonoids by the Wilstater reaction; tannins by the ferric chloride reaction; steroids and triterpenes by the Liebermann-Burchard reaction, and saponins by the foam index.

2.2.3. Spectrophotometric Determination of Total Polyphenols

The method of Wood et al [9] was used for the determination of total polyphenols. A volume of 2.5 mL of diluted (1/10) Folin-Ciocalteu reagent was added to 30 µL of extract. The mixture was kept for 2 minutes in the dark at room temperature, then 2 mL of calcium carbonate solution (75 g. L^{-1}) was added. The mixture was then placed in a water bath at 50 °C for 15 minutes, and rapidly cooled. Absorbance was measured at 760 nm, using distilled water as a blank. A calibration line was made with gallic acid at different concentrations (Ap-

pendix 1). Analyses were carried out in triplicate, and polyphenol concentration was expressed in grams per liter of gallic acid equivalent extract (mg. Eq GA/mL).

2.2.4. Spectrophotometric Determination of Total Flavonoids

The method of Marinova et al [10] was used for the determination of total flavonoids. In a 25 mL flask, 0.75 mL sodium nitrite ($NaNO_2$) 5% (w/v) was added to 2.5 mL extract. The mixture was supplemented with 0.75 mL aluminum chloride ($AlCl_3$) at 10% (w/v), then incubated for 6 minutes in the dark. After incubation, 5 mL sodium hydroxide (NaOH, 1N) was added and the volume made up to 25 mL. The mixture was shaken vigorously before being assayed on a Jasco V-530 spectrophotometer (JASCO, Japan). Readings were taken at 510 nm. Trials were carried out in triplicate. Flavonoid content was expressed in milligrams per liter of quercetin equivalent extract (mg EQ /mL).

2.2.5. Assessment of Antioxidant Activity by DPPH

The anti-free radical activity of "Ahoutou" extract was measured using the 2, 2-diphenyl-1- picrylhydrazyl (DPPH) inhibition test based on the method of Parejo et al [11]. This method assesses the extract's ability to bind free radicals by measuring the decrease in violet coloration due to the reduction of DPPH radicals. The dry aqueous extract of "Ahoutou" and ascorbic acid (reference antioxidant) dissolved in methanol at a concentration of 1 mg/mL were diluted to different increasing concentrations (0.025-0.05-0.1-0.2-0.3 mg/mL). DPPH was solubilized in absolute methanol to obtain a solution with a concentration of 6.34.10-5 M (0.0025 g DPPH in 100 mL methanol). The resulting solution was stored in a dark place. After 30 minutes incubation at room temperature, absorbance was read in a spectrophotometer at 517 nm against the blank sample. The free radical scavenging activity of the extracts tested, corresponding to the percentage inhibition (PI) of the DPPH radical, was calculated according to the following equation:

PI (%) = (A0 (Absorbance of blank) - A (Absorbance of extract) * 100) / (A0 (Absorbance of blank) Where A0 is the absorbance of the negative control, which contains DPPH and methanol without extract. The inhibition concentration IC50 (mg/mL), which corresponds to the concentration of the "Ahoutou" extract containing the reference antioxidant responsible for 50% inhibition of DPPH radicals, is used to assess the antioxidant power of the extract analyzed.

2.2.6. Evaluation of in Vitro Antiplasmodial Activity of Extracts

(i). Sample Collection

Patients attending the Formation Sanitaire Urbaine et Communautaire de Abobo Anonkoua Kouté, after a clinical diagnosis of uncomplicated malaria, were referred to the laboratory for biological diagnosis by Rapid Diagnostic Test (RDT). Patients with a positive RDT for *Plasmodium falciparum* mono- infection were fully briefed on the research project by our team. After obtaining the patient's consent, their blood is drawn by laboratory technicians. Blood samples are taken at the elbow by venipuncture, and collected in pre-identified EDTA tubes. The blood samples are then sent to the malaria laboratory of the Institut Pasteur de Côte d'Ivoire.

(ii). Preparation of Parasitized Red Blood Cells

Plasmodium falciparum isolates were collected under sterile conditions in EDTA-containing tubes from people suffering from uncomplicated malaria, and transported to the laboratory. Parasitized blood was washed three times with RPMI by centrifugation at 3,000 rpm for 5 minutes to remove plasma and white blood cells. At the same time, thick blood drops and blood smears were taken in duplicate to assess the parasitaemia of the samples. Parasitemia levels above 0.3% were diluted in healthy group O Rhesus- positive red blood cells to obtain parasitemia levels below 0.3%.

(iii). Preparation of Healthy Red Blood Cells

Group O, Rhesus-positive blood is collected from people who are not *Plasmodium* carriers. Blood samples were taken sterile on a violet tube containing the anticoagulant EDTA. Blood samples were washed three times with RPMI 1640 after centrifugation at 3,000 rpm for 5 minutes to remove plasma a n d white blood cells. Healthy red blood cells were stored at +4 ℃.

(iv). Inoculum Preparation

The inoculum was prepared from parasitized red blood cells and complete medium. Using four clinical isolates ANK137, ANK138, ANK139 and ANK140 with parasitaemia levels of less than 0.3%, nine 96-well plates were used, 3 per clinical isolate. For each plate, an inoculum volume of 12 mL was prepared. The volumes of the various inoculum components were 11.76 mL of complete medium and 240 µL of GRP for a hematocrit of 2%.

(v). Preparation of the Concentration Range of Crude Extracts

The stock solution of the "Ahoutou" extract to be tested was prepared at a concentration of 1 mg/mL. To do this, 10 mg of Ahoutou extract was dissolved in 10 mL of distilled water. The extract was vortexed for at least 10 minutes to ensure complete dissolution. The resulting solution was autoclaved at 121 ℃ for 15 minutes using the method described by Bolou et al [12]. In the first well (parasite growth control, i.e. no product), 100 µL of complete medium + 100 µL of parasitized globular suspension were added. In the second well (test well), 100 µL of test extract + 100 µL of parasitized globular suspension were added. In the other wells, 100 µL of complete

medium was added. From the test wells containing stock solutions with concentrations of 100 µL/mg extract or 800 ng/mL Dihydroartemisinin (DHA), successive dilutions were made to the half (1/2). Then, after homogenization, 100 µL of stock solution (second well) was taken and added to the next well, and so on. After homogenization of the last well, 100 µL are removed and discarded. Then, 100 µL of inoculum are added to the wells containing the plant extracts. This gave a concentration range of 50 µg/mL; 25 µg/mL; 12.5 µg/mL; 6.25 µg/mL; 3.125 µg/mL; 1.56 µg/mL; 0.78 µg/mL for the crude extracts. For the reference molecule Dihydroartemisinin (DHA), a concentration range of 800 nM; 400 nM; 200 nM; 100 nM; 50 nM; 25 nM; 12.5 nM was obtained. The culture plates were then enclosed in a candle bell and placed in an incubator maintained at 37 ℃. All manipulations were performed under a laminar flow hood.

(vi). Chemosensitivity Tests

Plasmodium falciparum isolates were donated in vitro using the method adapted from Trager and Jensen [12]. The principle of the in vitro blood-stage P. falciparum experiment is based on the fact that the parasites are placed in an experimental condition favorable to multiplication and under aseptic conditions. All manipulations were carried out under a laminar flow hood (Steril Gard®) to avoid bacterial contamination. Assays were performed in 96-well microplates filled with a fixed volume of parasitized red blood cells (parasitaemia < 0.3% and haematocrit 2%). Inhibition of erythrocyte schizogony (Plasmodium falciparum) was measured by the SYBR Green method. Test extracts at different concentrations were added to the wells in duplicate.

(vii). Preparation of SYBR Green

After 72 h incubation, the cultures were frozen and thawed. First, hemolysis of the culture was performed with a lysis buffer prepared by dissolving 1.21 g Tris-HCl in 350 mL distilled water. Next, a volume of 5 mL EDTA (0.5 M), 40 mg saponin and 400 µL Triton X- 100 were added. The volume was adjusted to 500 mL with distilled water. The resulting solution was filtered through a 0.22 µm millipore filter (Stericup Durapore ®) and stored at room temperature. For two experimental plates, a mixture consisting of 5 µL of SYBR Green I and 25 mL of lysis buffer was prepared. Lysis was performed by transferring 100 µL of the parasite suspension to a blank plate. To this 100 µL of parasite suspension, a volume of 100 µL of mixture was added. The plate was then incubated for one hour at room temperature, protected from light. Readings were taken using a spectrofluorometer at 535 nm after excitation at 485 nm.

(viii). Principle of the SYBR Green Method

This DNA fluorescence-based method is useful for drug screening, and is attractive due to the absence of nucleic acids in red blood cells. SYBR Green is widely used in in vitro studies. It is a DNA intercalant. After 72 hours of parasite incubation, the red blood cells were lysed by freezing and thawing the cultures. SYBR Green mixed with the lysis solution (called the mix) was then added to the culture. It inserts itself between the DNA bases and emits a fluorescence whose intensity is proportional to the DNA in the medium, which is proportional to the number of parasites. Fluorescence is then evaluated using a spectrofluorometer (DELL, FLx800, biotek) to monitor parasite growth.

(ix). Reading and Determination of Inhibitory Concentration 50 (IC) 50

Culture plates were incubated at 37 ℃ for 72 hours. Parasitemia was then assessed by the SYBR Green method. A mix containing 0.2 µL of SYBR Green per mL of lysis buffer was prepared. Lysis was performed by transferring 100 µL of mix (lysis buffer + SYBR Green) to a blank plate. To this 100 µL of mix, 100 µL of parasite culture was added. The plate was then incubated for one hour at room temperature, protected from light. Readings were taken on a spectrofluorometer (BIOTEK, FLX 800) at 528 nm after prior excitation at 485 nm. The ICs50 (extract concentration that inhibits 50% of parasite growth) and corresponding correlation coefficients were determined graphically, using WWARN's IVART (*In Vitro* Analysis and Reporting Tool) software according to Le-Nagard et al [13].

2.2.7. Statistical Analysis

Data were plotted using Graph Pad Prism 5.0 and Microsoft office Excel 2016. The mean value is accompanied by the standard error of the mean (Mean ± SEM). The difference between two values is considered significant when $P<0.05$. Statistical analysis of results was performed using analysis of variance (ANOVA). When a significant difference was observed, Tukey's and Dunnett's multiple comparison tests were performed.

3. Results and Discussion

3.1. Results

3.1.1. Phytochemical Screening

The qualitative phytochemical screening carried out on Ahoutou identified the main chemical groups it contains. Colorimetric analysis shows that Ahoutou contains polyphenols, flavonoids, saponins, tannins, alkaloids, sterols and terpenes.

Table 1. *Phytochemical characterization of the "Ahoutou" extract.*

Extract	Compounds					
	Polyphenols	Flavonoids	Saponins	Tannins	Alkaloids	Steroids and Triterpenes
Ahoutou	+	+	+	+	+	+

(+): present; (-): absent

3.1.2. Phenolic Compound Content

The phenolic compounds assessed were total polyphenols and total flavonoids. The quantitative analysis of Ahoutou total flavonoids was determined from the calibration curve of equation y = 0.2716x + 0.0004 with R^2= 0.96 established with increasing quercetin concentrations. These concentrations are expressed in mg quercetin equivalent per mL dry extract. Quantitative analysis of total polyphenols was based on the calibration curve equation y = 1.129x + 0.05437 with R^2 = 0.9935, established with increasing concentrations of gallic acid. Concentrations are expressed in mg gallic acid equivalents per gram of dry extract. Results for total flavonoids and total polyphenols are shown in Table 2.

The total flavonoid and total polyphenol contents of the "Ahoutou" recipe when still liquid (before steaming) are 0.65 ±0.03 mg. EQ/ mL and 3.2±0.06 mg. EAG/ mL, respectively. After concentration with rotavapor, the total flavonoid and total polyphenol contents of Ahoutou dry extract are 1.30 ± 0.10 mg. EQ/ mL and 7.70±0.82 mg. EAG/ mL respectively. The results also show that flavonoid and polyphenol contents in Ahoutou dry extract are higher.

Table 2. *Total flavonoids and total polyphenols content of Ahoutou.*

Extract	Flavonoid content (mg. EQ/mL)	Polyphenol content (mg. EAG/mL)
Ahoutou	0,65 ±0,03	3,2±0,06
	1,30 ±0,10	7,70 ±0,82

Yield results are expressed as mean ±SD, n=3 for extract

3.1.3. Antioxidant Activity: DPPH Reduction

Antioxidant activity was tested using the DPPH method. Determination of the concentration of Ahoutou and ascorbic acid required to reduce 50% of DPPH free radicals (CI50) from the curves yielded values of 1.46 ± 0.03 mg/mL for "Ahoutou" extract and 0.25 mg/mL for ascorbic acid (Table 3).

Table 3. *Antioxidant capacity of Ahoutou according to DPPH tests.*

Compounds	% Inhibition IC50 (mg/mL)
Ahoutou	1,46 ±0,03
Vitamin C	0,25 ±0,00

3.1.4. Antiplasmodial Activity

The antiplasmodial activity of the "Ahoutou" extract was expressed as a 50% inhibitory concentration (CI50). Ahoutou was tested on 4 clinical isolates and on the reference *Plasmodium falciparum* strain Dd2. The results are shown in Table 4. Analysis of the table shows that Ahoutou exhibited significant antiplasmodial activity on clinical isolate ANK137 with an IC50 of12.86 µg/mL, and moderate antiplasmodial activity on clinical isolates ANK138, ANK139 and ANK140 with IC50 of 23.55 µg/mL, 22.72 µg/mL and 18.92 µg/mL respectively. When tested on the reference strain of *Plasmodium falciparum* Dd2, Ahoutou showed moderate anti-plasmodial activity (15<IC50 <50 µg/mL).

Table 4. Antiplasmodial activity of Ahoutou on clinical isolates and reference strain.

Extracts	CI50 (µg/mL)				
	Clinical isolates				Reference strain
	ANK137	ANK138	ANK139	ANK140	Dd2
Ahoutou	12,86	23,55	22,72	18,92	23,42
DHA (nM)	0,59	0,22	0,66	5,59	2,31

3.2. Discussion

The results of phytochemical screening show that the extract from this recipe contains flavonoids, polyphenols, tannins, saponins, sterols and terpenes.

Phytochemical screening of *Azadirachta indica* by Abalaka et al [14] revealed the presence of flavonoids (quercetin), saponins, polyphenols, tannins and triterpenes. Alkaloids, glycosides, carbohydrates, trace saponins, terpenes, tannins, flavonoids and phenolic compounds were found in *Cymbopogon citratus* [15], then in *Psidium guajava*, Biswas et al. [16] showed that the aqueous extract of *Psidium guajava* leaves could contain phenols, tannins, saponins, terpenoids, flavonoids and glycosides. The presence of different chemical groups in the Ahoutou recipe therefore explains the use of these plants in the production of Ahoutou. On the other hand, the results seem to indicate a low presence of total phenols and flavonoids in the Ahoutou recipe. The results obtained from the quantification of total flavonoids and total polyphenols show that the Ahoutou recipe is less rich in total polyphenols and total flavonoids, as predicted by the phytochemical screening. Most antioxidant molecules are phenolic compounds [17]. Given the involvement of various factors such as the physico-chemical properties of the molecules, it is recommended to use different tests to confirm antioxidant activity [18]. We therefore chose to use the DPPH test. Evaluation of antioxidant activity *in vitro* showed that the Ahoutou recipe was capable of reducing DPPH free radicals. This reducing and anti-free radical power is based on the phenolic compound content of the Ahoutou recipe. Indeed, polyphenols are compounds with antioxidant power due to their redox properties [19]. These properties enable them to neutralize free radicals by donating electrons or protons [20], and to block the free radical reaction chain by transferring hydrogen atoms [21]. This antioxidant activity of polyphenols is often exploited to prevent and treat diseases linked to oxidative stress. According to Han et al [22], phenolic compounds possess venotonic and vasculoprotective properties that could be beneficial in preventing vascular damage in hypertensive patients. The antiplasmodial activity of Ahoutou and the current reference molecule in malaria treatment, Dihydroartemisinin (DHA), was assessed *in vitro* on clinical isolates and on the chloroquine- resistant *Plasmodium falciparum* reference strain Dd2, using the SYBR green I fluorescence-based assay. This method represents an inexpensive and relatively simple approach from a technical point of view [23]. The choice of clinical isolates was motivated by the need to work with strains present in the target population. But also because the determination of antimalarial activity on reference strains of *Plasmodium falciparum* also poses the problem of the validity of *in vitro* tests. Indeed, the various strains used are adapted to laboratory culture and may no longer correspond to the reality in the field. This is why it seemed worthwhile to evaluate the activity of extracts in general, and Ahoutou and the reference molecule in particular, on isolates from malaria patients. Our results showed that Ahoutou has activity on chloroquine-resistant strains according to the classification scale for natural substances established by Beourou et al. [24]. According to these authors, a plant extract is said to have a promoting effect if the IC50 is between 5 µg/mL and 15 µg/mL (5<CI50 <15 µg/mL) and a moderate effect if its IC50 is between 15 µg/mL and 50 µg/mL (15<CI50 <50 µg/mL). The Ahoutou recipe showed a promoter effect on one isolate (12.86 µg/mL) and a moderate effect on three clinical isolates (23.55 µg/mL, 22.72 µg/mL and 18.92 µg/mL), then on the reference strain Dd2 (23.42 µg/mL). Phytochemical screening of Ahoutou also revealed the significant presence of polyphenols, flavonoids and traces of alkaloids. Indeed, the cumulative presence of compounds such as polyphenols and alkaloids in the Ahoutou recipe (a blend of three plants) could have a synergistic effect on *Plasmodium falciparum* [25].

4. Conclusion

In this part of the work, the aim was to provide scientific evidence of the therapeutic activity of a plant- based preparation used in the treatment of malaria. This preparation, called "Ahoutou" by its designer, is used in humans, but has not previously been the subject of preclinical studies on malaria patients to assess its therapeutic properties. According to the qualitative analysis, Ahoutou contains secondary metabolites, notably phenolic compounds. Ahoutou also possesses signif-

icant antiradical and antioxidant activity thanks to the presence of these compounds. It also has a moderate anti-plasmodial effect on *Plasmodium falciparum*.

However, this activity could be enhanced by modern extraction tools or techniques (processes) to make it more effective against malaria.

Abbreviations

ACT Artemisinin-based Combination Therapy
ANK Anonkoua Kouté
RDT Rapid Diagnostic Test
DPPH 2, 2-diphenyl-1- picrylhydrazyl

Author Contributions

Gouegoui Serge-Pacome Bohui: Conceptualization, Investigation, Methodology, Writing – original draft
Sylvain Beourou: Validation, Visualization
Djibiliour Sanogo: Resources, Visualization
Jean David N'guessan: Supervision, Validation
Koffi Barthelemy Attioua : Supervision
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Conflicts of Interest

The authors declare no conflicts of interest.

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Research Fields

Gouegoui Serge-Pacome BOHUI: organic chemistry, chemistry of natural substances, phytochemistry, pharmacology, theoretical chemistry

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