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Full Length Research Paper

Biological activities of *Peganum harmala* leaves

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Ethyl acetate, chloroform, butanol and methanol extracts of the leaves of *Peganum harmala* were tested for antibacterial, antioxidant and antiviral activities. The antibacterial activity was evaluated by the determination of minimum inhibitory concentration (MIC) using the solid medium technique. The antiviral activity was evaluated against human cytomegalovirus (HCMV) strain AD-169 (ATCC Ref. VR 538) and Coxsackie B virus type 3 (CoxB-3) using diagnostic method 'shell-vial' culture. The antioxidant activity was evaluated using ammonium thiocyanate method. Among tested extracts, methanol and chloroform extracts displayed a higher antibacterial activity against gram-positive than gram-negative bacteria. The methanol extract demonstrated the highest antioxidant activity and good antiviral activity against HCMV.

Key words: *Peganum harmala*, antimicrobial, antiviral, antioxidant, activities, leaves.

INTRODUCTION

Herbal medicine represents one of the most important fields of traditional medicine all over the world. Medicinal plants are traditionally used for the treatment of pain. Formation of free radicals may play an important role in the origin of life and biological evolution, implying their beneficial effects on aging of organisms and cancer promotion (Ashok and Ali, 1999). The interest in natural antioxidants, especially of plant origin has greatly increased in recent years (Jayaprakasha et al., 2000). Natural antioxidants can protect the human body against free radicals that may cause some chronic diseases (Kinsella et al., 1993; Lai et al., 2001). The researches conducted on the antioxidant activities of some plants as natural antioxidants generally focused on the herbs and aromatic plants (Miliauskas et al., 2004; Pizzale et al., 2002).

Peganum harmala L. (Syrian rue) is a wild growing flowering plant belonging to the Zygophylaceae family and is considered an important medicinal plant. The seeds are known to possess hypothermic and hallucinogenic properties (Kuhn and Winston, 2000; Lamchouri et al., 1999).

P. haramala was shown to possess antihelmitic, lactogogue, antispasmodic, antipyretic, abortifient, emetic and emmenagogue properties (Chopra et al., 1958). Thus, the objective of this study was to determine the antimicrobial, antiviral and antioxidant properties in extracts obtained from the leaves of *P. harmala* grown in Tunisia.

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Abbreviations: FTC, Ferric thiocyanate; **DMSO,** dimethylsulphoxide; **PBS,** phosphate buffered saline; **HCMV,** human cytomegalovirus; **CoxB-3,** coxsackie B virus type 3; **MICs,** minimum inhibitory concentrations; **BHI,** brain heart infusion; **LPI,** lipid peroxidation inhibition.

MATERIALS AND METHODS

Plant materials

Plants materials were collected in 2008 in Kerker (Tunisian centre). The plant was identified by Pr. Mohamed Chaieb, a botanist in the University of Science (Sfax, Tunisia). A voucher specimen was deposited in the herbarium of our laboratory.

Extraction

Five hundred gram (500 g) of dried *P. harmala* leaves were extracted with methanol (MeOH) through maceration for five days. The resulting extract was evaporated at reduced pressure to obtain a green residue (231 g) which was successively fractionated with petroleum ether, chloroform and ethyl acetate, and these fractions were used for biological tests.

Total phenolic content

The polyphenol content of the extracts were determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method (Singleton and Rosi, 1965), calibrating against catechin standards and expressing the results as mg catechin equivalents (CAE) g–1 extract. Data presented are the average of three measurements.

Determination of antimicrobial activity

Microorganisms

The microorganism strains used in the biological assays are listed in Table 1. Different American-Type Cell Culture (ATCC) reference bacteria and fungi as well as clinical isolates including methicillin susceptible (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA) strains were used.

Determination of minimal inhibitory concentration

Various concentrations (from 1 to 10 $mgml^{-1}$) of extracts of *P. harmala* leaves were used to determine the antimicrobial activity. Overnight, broth cultures were adjusted to yield approximately 1 x 10^6 $CFUml^{-1}$ of bacteria or yeast. The minimum inhibitory concentrations (MICs) were determined on brain heart infusion (BHI) agar plates (Bio-Rad, Marne la Coquette, France) by a standard method (NCCLS, 1997) with a Steers-type replicator device that delivered approximately 10^4 bacteria per spot. One milliliter (1 ml) of each of the extracts previously dissolved in 10% dimethyl sulfoxide (DMSO) were mixed for each concentration with 19 ml of BHI agar at 40°C and poured over Petri dishes. The resulting DMSO concentration was approximately 0.5%. Plates containing only medium or medium with 0.5% DMSO were used as controls to ensure that DMSO did not affect growth standard antibiotics (oxacilin, amoxicillin, ticarcillin and cefotaxim were used in order to control the sensitivity of the tested microorganism). After 18 h incubation at 37°C, the MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each microorganism. Each test was carried out in triplicate. The microorganisms tested in this study were provided from the European Hospital of George Pompidou (HEGP) (France).

Antiviral activity

Cell toxicity assay

The cytotoxic activity was determined by the MTT assay (Polydoro et al., 2004).

Titration of viruses

Human cytomegalovirus (HCMV) strain AD-169 (ATCC Ref. VR 538) and coxsackie B virus type 3 (CoxB-3) were used for the antiviral activity. Serial 10-fold virus dilutions (10^{-1}, 10^{-5}) were prepared in MEM-D containing 2% FCS, inoculated into confluent cells in quadruplicate wells of 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 – 5 days. When a cytopathic effect (CPE) in the virus-infected cells were observed microscopically, virus titers were expressed as 50% tissue culture-infective dose (TCID50) and were determined by the method of Reed and Muench (1938) antisera. The mouse monoclonal antibodies directed against the immediate-early CMV 76kD antigen (clone E-13, Ref. 11-003, ARGENA-Biosoft, France) was diluted 1:50 in PBS. The same source provided the goat-anti-mouse FITC-conjugated antibody (Ref. 50- 012).

Antiviral activity test

The test used in this paper is a modification of the generally used 'shell-vial' culture method for the early detection of the CMV infection (Alpert et al., 1985). The diagnostic method is sensitive (Pepin et al., 1991) and quantitative (Mazeron et al., 1991). The antiviral activity of the extracts of the plants was diluted in MEM-D, then the virus was added and centrifuged for 1 h at 1000 g. After centrifugation, the wells were incubated for 48 h at 37°C, and the viral antigens were revealed by immunofluorescence. The culture medium was aspirated and the cells were infected with 600 ml of the virus dilution followed by centrifugation at 1000 g for 1 h. After centrifugation, the mixture was incubated for 48 h at 37°C, then the viral antigens were revealed by immunofluorescence.

The immunofluorescence detection of the viral antigens was common for the above procedures and was as follows: at the end of incubation, the content of the wells were aspirated and the cells were fixed with cold methanol at 20°C for 10 min. The methanol was aspirated and 250 ml of anti immediate-early antibody was added and incubated for 45 min at 37°C in a humidified chamber. At the end of this time the wells were washed three times for 5 min with phosphate buffered saline (PBS). Then the FITC labeled anti-mouse antibody was incubated for 40 min and finally washed under the same conditions three times as previously done. After that, the cells were observed under an immunofluorescence microscope. The number of infected cells were compared with a known number of viral particles (average of 1000 particles) in the presence or absence of the plant extract. Because the extracts were dissolved in dimethylsulphoxide (DMSO) which positively affects the infectivity of the viruses (Li and Fong, 1990), DMSO was also included in the wells without any extract. The antiviral activity was determined by counting the number of cells infected by the virus (presenting fluorescent nuclei) in comparison with the number of infected cells in the reference well (no presence of any antiviral agent). The procedure was selected to compare the antiviral activity of the extracts and references ganciclovir and ribavirine. The reference well contained cells infected with the virus without any antiviral agent. The above described experiments were done three to four times.

Antioxidant activity by ammonium thiocyanate method

The lipid peroxidation inhibition activity was measured in a linoleic acid emulsion system according to the methods of Osawa and Namiki (1985). Briefly, a sample (1.3 mg) was dissolved in 10 ml of 50 mM phosphate buffer (pH 7), and a solution of 0.13 ml of linoleic acid and 10 ml of 99.5% ethanol was added. Then the total volume was adjusted to 25 ml with distilled water. The mixture was incubated at 40°C for 5 days in a dark room, and the degree of oxidation was evaluated by measuring the ferric thiocyanate (FTC), according to Mitsuda et al. (1966). A total of 100 ml of the oxidized linoleic acid solution described above was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 0.02 M

Table 1. Antibacterial activity of leaves extracts of *P. harmala*.

Sources	MIC (mgml^{-1})				MIC of antibiotics (µg/ml) [a)]				
	Meth	But	AE	Ch	OXA	AMX	TIC	CTX	AB
Gram negative bacteria									
Escherichia coli ATCC25922	2.5	5	5	5	ND [e)]	8	ND	0.094	ND
Escherichia coli HEGP402	2.5	5	5	5	ND	(2;>256)	ND	0.094	ND
Klebsiella pneumoniae HEGP8326.	5	5	5	5	ND	>256	ND	0.064	ND
Enterobacter cloacae HEGP4102	5	5	5	5	ND	>256	ND	(0.125; 0.32)	ND
Serratia marcescens HEGP1002	5	5	5	5	ND	>256	ND	>256	ND
Acinetobacter baumannii HEGP1003	5	5	5	5	ND	ND	1	ND	ND
Gram positive bacteria									
Bacillus subtilus ATCC 6633	2.5	1.25	1.25	0.256	ND	0.064	ND	3	ND
Staphylococcus aureus ATCC25923	1.25	5	5	1.25	0.19	ND	ND	ND	ND
Staphylococcus aureus ATCC29213	1.25	5	5	1.25	0.25	ND	ND	ND	ND
Staphylococcus aureus MSSA [b)] HEGP8064	0.512	5	5	0.512	(0.38-0.75)	ND	ND	ND	ND
Staphylococcus aureus MRSA [c)] HEGP4945	0.512	5	5	0.512	[8-16]	ND	ND	ND	ND
Streptococcus pyogenes HEGP1004	0.512	0.512	5	0.512	ND	ND	ND	ND	ND
Streptococcus agalactiae HEGP1005	0.256	1.25	1.25	0.256	ND	0.094	ND	[0.032; 0.064)	ND
Enterococcus faecalis ATCC29212	1.25	5	5	2	ND	1	ND	ND	ND
Enterococcus faecalis HEGP7980	1.25	5	5	2	ND	1	ND	ND	ND
Enterococcus faecium HEGP1007	2.5	5	1.25	0.512	ND	(32;>256)	ND	ND	ND
Corynebacterium spp HEGP1006	2.5	5	5	0.256	ND	>256	ND	ND	ND
Yeasts									
Candida glabrata ATCC 90030	2.5	2.5	2.5	2.5	ND	ND	ND	ND	0.5
Candida albicans ATCC 90028	2.5	2.5	2.5	2.5	ND	ND	ND	ND	0.5
Candida parapsilosis ATCC 22019	2.5	2.5	2.5	2.5	ND	ND	ND	ND	0.5
Candida kreusei ATCC 6258	2.5	2.5	2.5	2.5	ND	ND	ND	ND	0.5

AE: Ethyl acetate; But: butanol; Ch: chloroform; Met: Methanol. Values are mean of triplicate determinations (n = 3) ± standard deviation.
a: Minimal inhibitory concentration of antibiotics; values given as µg/ml
b: strain sensitive to methicillin
c: strain resistant to methicillin

ferrous chloride solution in 3.5% HCl. After stirring for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm using a spectrophotometer. α-Tocopherol was used as a reference and distilled water as a control. The lipid peroxidation inhibition (LPI) percentage (%) was calculated as:

LPI = [1 - (Absorbance of sample / Absorbance of control)] x 100.

RESULTS

Total phenolic content

The results indicate that among the tested extracts, methanolic and butanolic extracts had the highest total phenolic content (112.5; 63 mgCEg^{-1}) Figure 1. The ethyl acetate extract had the lowest total phenolic content (25.7 mg CEg^{-1}). The results indicate that the efficiency of methanol for the extraction of total phenolic compounds was the highest.

Antibacterial activity

The results of the antibacterial and antifungal activities of *P. harmala* leaves extracts are presented in Table 1. The chloroformic, ethyl acetate, butanolic and methanolic extracts of *P. harmala leaves* all displayed good anti-fungal activity with MIC values of 2.5 mgml^{-1}. Chloroformic and methanolic extracts showed important antibacterial activity against gram positive bacteria than gram negative bacteria with MIC values ranging between 0.251 and 2.5 mgml^{-1} (Table 1). Butanolic and ethyl acetate extracts showed antibacterial activity against gram positive bacteria with MICs values ranging between

Table 2. Antiviral activity of leaves extracts of *P. harmala*.

Extracts	Concentration (µg/ml)	Antiviral activity (%)	
		HCMV	Cox B3
Petroleum ether	100	0	0
	50	0	0
	25	0	0
Ethyl acetate	100	65	24
	50	23	16
	25	19	12
Chloroform	100	51	16
	50	32	12
	25	20	10
Butanol	100	75	31
	50	45	25
	25	19	23
Methanol	100	95	52
	50	85	43
	25	80	21
Ganciclovir[b]	3.3	51	ND
	1.7	31	ND
	0.8	20	ND

b: Ganciclovir, which is clinically used anti-HCMV drug, was used as positive controls in the antiviral activity.

0.512 and 5 mgml^{-1} but this activity is lower than the other extracts.

Antiviral activity

The antiviral activity of the extracts of leaves of *P. harmala* in different concentrations in comparison with that of ganciclovir are summarized in Table 2. According to this table, all extracts had anti-HCMV activity in different concentrations except petroleum ether extract which did not have any antiviral activity. The most active extract seemed to be methanol extract. The active concentrations of the different extracts ranged from 25 mgml^{-1} (80% activity) to 100 mgml^{-1} (95% activity). As shown in Table 2, all extracts did not exhibit important anti-Cox B3 viruses, only methanol extract manifested moderate antiviral activity against HCMV.

Antioxidant activity by ammonium thiocyanate method

The antioxidant effects of the extracts from the leaves of *P. harmala* and α-tocopherol on the peroxidation of linoleic acid were investigated and the results are presented in Table 3. In the ammonium thiocyanate system, oxidation of linoleic acid was effectively inhibited by the methanol extract (75.9 ± 0.3), butanol and ethyl acetate extracts (67.8 ± 0.48 and 60.8 ± 0. 3, respectively) after incubation for 120 h (5 days). As seen in Figure 2, methanol extract displayed higher antioxidant activity when compared with positive control α-tocopherol (80.12 ± 0.4).

Table 3. Antioxidant activity of the leaves of *P. harmala* extracts by the ammonium thiocyanate method.

Extract	Inhibition of peroxidation (%)
Ch	50 ± 0. 48
AE	60.8 ±0.3
But	67.8 ±0.43
Met	75.9 ±0.5
α-Tocopherol	80.6± 0.4

AE: Ethyl acetate; But: butanol; Ch: chloroform; Met: Methanol. Values are mean of triplicate determinations (n =3) ± standard deviation.

DISCUSSION

To our knowledge, this study represents the first demonstration of antibacterial antiviral and antioxidant activity of *P. harmala* leaves extracts.

In the present study, we determined the phenolic composition of *P. harmala* leaves extracts from an arid region in Tunisia. Differences in polarity of the antioxidative components may explain why extraction yield and antioxidant activity of the extracts differ. The solubility of phenolic compounds are governed by the type of solvent used and the degree of polymerization of phenolics. In previous work done by Edziri et al. (2009), the phenolic content in aerial part of *P. harmala* is higher than the phenolic content in the leaves. In the present work, the methanol extract showed the highest amount of the polyphenols. For this purpose, methanol was recommended and frequently used for the extraction of phenolics (Abreu et al., 2005; Galvez, et al., 2005).

In comparison with other works done by Marzouk et al. (2009), we found that the methanol and chloroform extracts of *P. haramala* had good antibacterial activity against gram positive bacteria than the extracts of *Citrullus colocynthis* which have weaker antibacterial activity that ranged from 0.41 to 3.25 mgml^{-1}. In addition, the methanol extract of *Bacillus subtilis* showed antibacterial activity against *S. aureus* at MIC 512 mgml^{-1} (Basile et al., 1998). Same result was found in our work with methanolic and chloroformic extract of *P. haramala* leaves.

Antibiotic resistance is increasing worldwide in both outpatients as well as hospitalized patients. In the last two decades, the problem has escalated as the prevalence of antibiotic-resistant bacteria has increased and multi-drug-resistant strains have emerged in many species that cause disease in humans. There are no

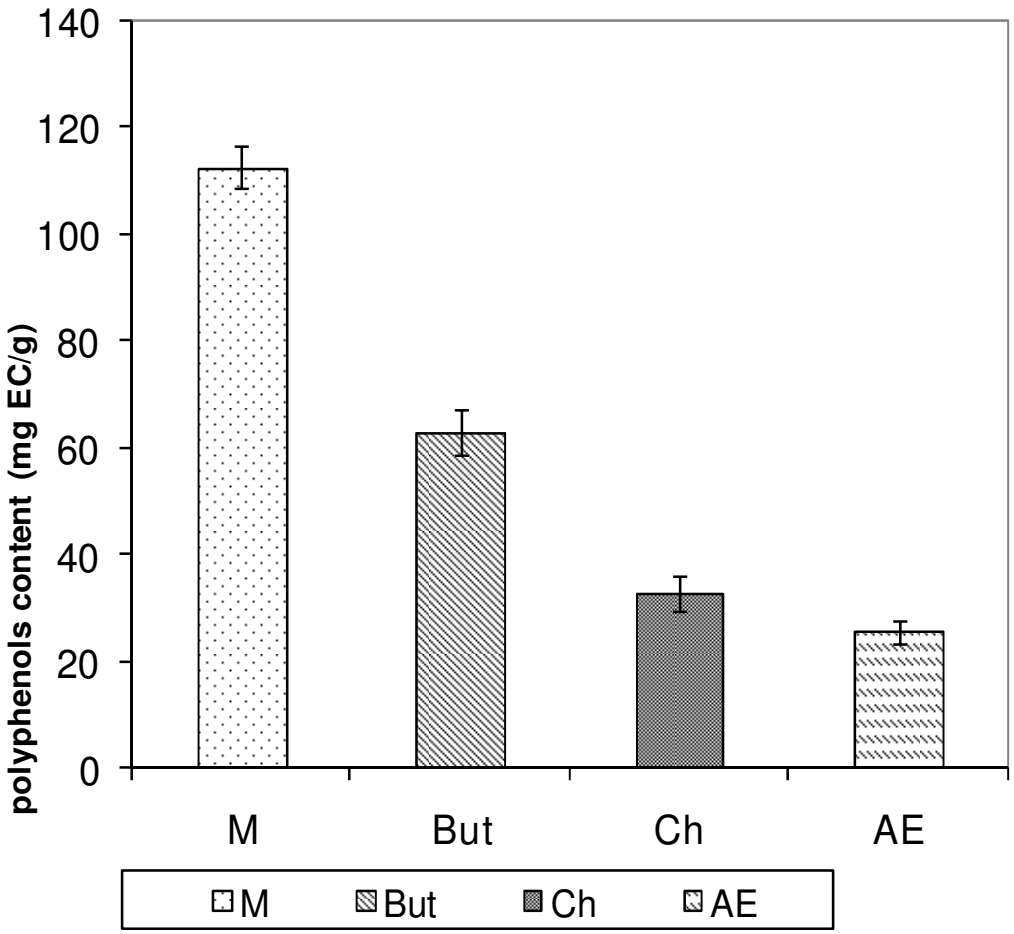

Figure 1. Polyphenols contents of *P. harmala* leaves extracts AE: ethyl acetate; But: butanol; Ch: chloroform; Met: Methanol

treatments available for infections caused by many of the antibiotic-resistant bacteria, and resistance to commonly used antibiotics is steadily increasing. Our finding shows that methanolic and chloroformic extract displayed higher antibacterial activity against gram-positive than gram-negative bacteria which are greater than other extracts. The reason for higher sensitivity of the gram-positive than gram-negative bacteria may be due to the differences between their cell wall compositions. The gram-positive bacteria contain an outer peptidoglycan layer, which is an ineffective permeability barrier (Nikaido and Vaara, 1985).

The observed antibacterial activity of the extracts of aerial part of *P. harmala* might also be attributed to the high quantity of polyphenols, which are known to possess efficient antibacterial activity (Scherrer and Gerhar, 1971). Cowan (1999) showed that several classes of polyphenol such as phenolic acids, flavonoids and tannins serve as plant defence mechanism against pathogenic microorganisms.

The antiviral activity of methanolic extract is probably due to the high phenolic content and the presence of polar substances such as flavonoids and tannins which are known to possess antiviral activity (Abidi and Ali, 1999; Fukuchi et al., 1989; Guillen and Manzanos, 1998; Kujumgiev et al., 1999).

Recently, numerous reports described antioxidants and compounds with radical scavenging activity present in fruit, vegetable, herb and cereal extracts (Cotelle et al., 1996; Hou et al., 2005). It seems that the antioxidant activity seen with the ammonium thiocyanate method for methanol, butanol, ethyl acetate and chloroform extracts may be predominantly related to the presence of the phenolic compounds such as flavonoids and tannins present in these polar extract.

The correlation level between the phenolic content and antioxidant activity between the plant organs is an interesting aspect, which supports the hypothesis that the former compounds contribute directly to antioxidant activity. Thus our findings show that leaves extracts of *P. harmala* also possess antibacterial, antiviral and anti-oxidant properties, which may be of beneficial when used therapeutically in humans.

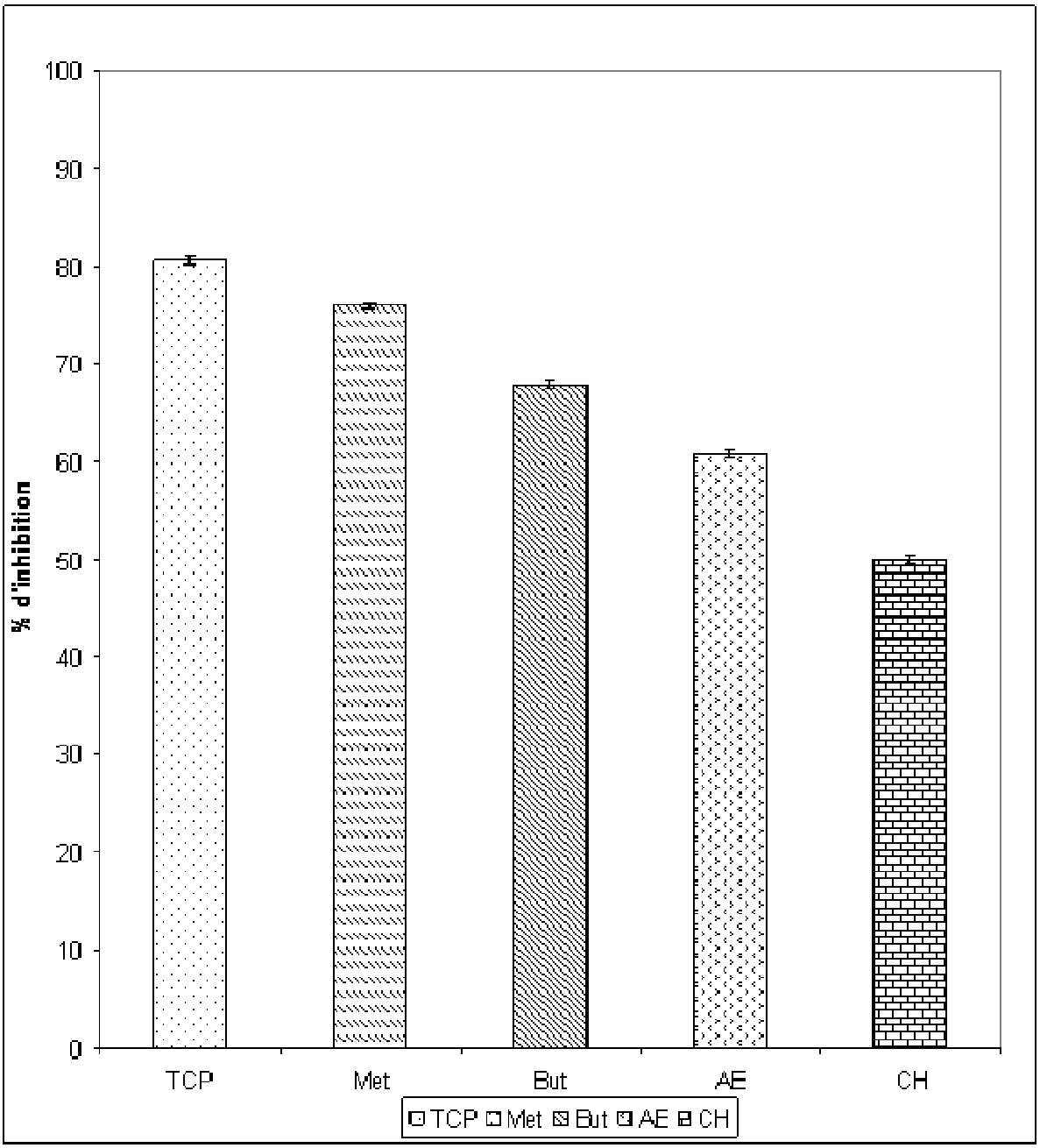

Figure 2. Antioxidant activity of the leaves of *P. harmala* extracts by the ammonium thiocyanate method. AE: ethyl acetate; But: butanol; Ch: chloroform; Met: Methanol; TCP: α-tocopherol.

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