Full Length Research Paper

Extra- and intracellular antimycobacterial activity of *Arbutus unedo* L.

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Accepted 17 January, 2012

This study aims to evaluate extra- and intracellular antimycobacterial activity of *Arbutus unedo* L. leaves-extracts. We tested the aqueous and ethanol extracts of *Arbutus* leaves effect against three mycobacteria growth, and showed that aqueous extract and the part I of ethanol extract have a remarkable extracellular antimycobacterial activity. The MIC (Minimum inhibitory concentration) of the part I of ethanol extract is 5.59 ± 0.69 mg/ml for *Mycobacterium aurum* A + and 6.02 ± 0.76 mg/ml for *Mycobacterium smegmatis* MC₂ and *Mycobacterium bovis* PPI. The part I of *arbutus* ethanol-extract, used at 6.02 ± 0.76 mg/ml, has a bactericidal effect on *M. smegmatis* MC₂ located within the rat peritoneal macrophages.

Key words: Antimycobacterial activity, *Arbutus unedo* L., rat peritoneal macrophages.

INTRODUCTION

The infectious disease tuberculosis is engendered by several species of *Mycobacteria* (Asgharzadeh and Kafil, 2007) including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium avium* and *Mycobacterium leprae* that are intracellular, Gram-positive, non-motile, rod-shaped, and obligate aerobic pathogens of higher vertebrates (Chakraborty, 2004).

Pathogenic mycobacteria agents are particularly ingenious, as they are able to survive, and even replicate within macrophages that are designed specifically to kill bacteria. They are exceptional in the duration and persistence of this interaction, seeing that the mycobacterial phagosome does not fuse with lysosomes (Armstrong and Hart, 1975; Frehel et al., 1986; Hart and Young, 1991). Latent infection as a main obstacle to controlling tuberculosis results from the survival of *M.*

tuberculosis in macrophage (Houben et al., 2006; Zahrt, 2003).

In addition to HIV-infection spreading, tuberculosis progress especially in countries with insuitable health care-systems providing an expensive and long-term treatment (World Health Organisation, 2008; Zager and McNerney, 2008). The emergence of multiple drug resistant (MDR) strains is a further crisis, including extremely drug resistant (XDR) *M. tuberculosis*-strains (Jones et al., 2008).

Even though the number of tuberculosis-related deaths appears to have stabilized at about 2 million per annum, the incidence of new infections increase largely because of HIV epidemic (Gutierrez-Lugo and Bewley, 2008). There would be many challenges to eradicate tuberculosis, including latency and drug resistance (Gutierrez-Lugo and Bewley, 2008). New purposes for novel anti-tuberculosis drugs need to be identified, as emergent MDR and XDR *M. tuberculosis*-strains. Although the complete genome sequences of *M. tuberculosis* H37Rv (Cole, 1998) and two other members

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of the *M. tuberculosis* complex (Cole, 2002) are available, it is likely to be many years before exploitation of this information yields novel therapeutics (Cole, 2002).

Plants and other natural materials may prove to be valuable sources of useful new antimycobacterial drugs. A number of studies have revealed the existence of a range of natural products with promising activity against mycobacteria (Newton, 2000; Cantrell, 2001).

Strawberry tree, *Arbutus unedo* L. (*Ericaceae*), is a typical species of Mediterranean fringe and climate, but today it is also cultivated in many other regions such as the near East and Transcaucasia (Seidemann, 1995). Previous studies indicate that leaves of *Arbutus unedo* contain several phenolic compounds like tannins, flavonoids, phenolic glycosides, lipids and vitamin E (Chevolleau et al., 1992; Fiorentino et al., 2007; Kivçak and Mert, 2001; Pabuçcuoğlu et al., 2003). This melliferous plant has a medicinal properties, namely the leaves and bark (Rocha-Afonso, 1991). In the Oriental Morocco, the leaves are frequently used in the traditional medicine system as a natural remedy for hypertension and diabetes (Ziyyat et al., 1997). Beside this effect, other properties were reported in the literature. Experimental investigations have shown that the ethanol and methanol extracts of *Arbutus unedo*-leaves present a potent antioxidant activity (Pabuçcuoğlu et al., 2003). The ethanol extract can also be a promising antileishmanial agent (Kivçak et al., 2009), and the ethyl acetate extract of strawberry leaves show an anti-trichomonad effect (Ertabaklar et al., 2009). Moreover, the aqueous extract of the plant exhibited antihypertensive and vasorelaxant activities, and they inhibited *in vitro* rat platelet aggregation (Mekhfii et al., 2006; Ziyyat et al. 2002; Ziyyat and Boussairi, 1998).

In the present study, we are investigated an extra- and intracellular antimycobacterial activities of *Arbutus unedo*-leaves extracts.

MATERIALS AND METHODS

Plant material

Arbutus unedo leaves were collected in October, 2009 from the Imouzzar Kandar surrounding-area (33°43'40"N-5°0'43"W).

Specimens of this species are gathered in the National Institute of Medicinal and Aromatic Plants, Sidi Mohamed Ben Abdellah University, Fez (exsiccata INP789).

The leaves were dried in the open air shade. They were then stored in paper bags, at a room temperature and kept away from humidity till the date of extraction.

Antimycobacterial strains and growth conditions

Three non-pathogenic mycobacterial strains were used during this study. Two fast growing species are *M. aurum* A+ and *M. smegmatis* MC₂ and one slow growing is *M. bovis* PPI". This strain is used as a model to evaluate the effect of active substances on the growth of *M. tuberculosis* (Newton et al., 2000, 2002). *M. smegmatis* and *M. aurum* have been shown to have similar profile of sensitivity to anti-tuberculosis drugs (Chung et al., 1995; Mitscher

and Baker, 1998). *M. bovis* is more closely resembled to *M. tuberculosis* than *M. aurum* and *M. smegmatis* seeing that *M. tuberculosis* and *M. bovis* are both slow growing organisms (Newton et al., 2002).

These bacteria are cultivated at 37°C on Sauton medium (Allen 1998; Papa et al., 1987). The mycobacteria were kindly provided by Dr. Suzana David Dr. S. David (Centro de Tuberculose e Micobactérias, Instituto Nacional de Saúde Dr. Ricardo Jorge Delegação do Porto, Portugal).

Preparation of plant extracts

(i) Aqueous extract: dried and powdered leaves from *Arbutus unedo* (15 g) were plunged in ebullient water during 1 h (infused) or in ebullient water and heated during 15 and 30 min (decocted). The extracts were then filtered.

(ii) Ethanol extract: dried and powdered leaves from *Arbutus unedo* (15 g) were extracted by maceration in 50 ml of ethanol during 24 h. This extract was then filtered and the organic solvent evaporated using a rotary-evaporator at 40°C to yield 1035.3 ± 94.2 mg of crude extract. The residue obtained was prepared by first dissolving in (DMSO) (Newton et al., 2002) and diluting with a liquid Sauton medium or RPMI-1640 culture medium of macrophages and then further filtered. 598.5 ± 73.9 mg of crude extract are soluble in medium culture (part I) and 436.8 ± 26.2 mg not soluble in the medium culture filter results (part II). The volume of liquid medium added was differed according to a concentration of extract desired. The DMSO ratio should be inferior or equal 2%. Control experiments showed that a final concentration of DMSO (2%) did not affect the growth of antimycobacterial strains used. The extracts were neutralized and sterilized by filtration through a 0.45 µm membrane filter.

Extracellular antimycobacterial activities of aqueous and ethanol plant extracts

The test of the extracellular antimycobacterial activity was realized by the extracts incorporation in the culture medium according to the agar diffusion and agar dilution and broth dilution assays.

(i) Agar dilution assay: aqueous and ethanol extract (part I) are added in the culture medium Sauton-agar (Allen, 1998; Papa et al., 1987) to reach a final concentration of 160 mg of dry matter per ml of medium. This concentration is relatively elevated. Indeed, using a less concentration could hide the antimycobacterial effect of plant extracts of which the active principle is present in very faint quantity. Besides, several anterior investigations have used concentrations that are superior or equal to 100 mg/ml in dry matter (Ahmad and Beg, 2001; Cos et al., 2002; Olila et al., 2001). Aliquots of 100 µl of mycobacterial cultures of *M. smegmatis* MC₂ and *M. aurum* A+ and *M. bovis* IPP (10⁶ UFC/ml) were then spread over the medium thus prepared and incubated at 37°C. The results are read every day during 6 days for rapidly growing mycobacteria and after 21 to 30 days of incubation for *M. bovis* PPI. This test was repeated 4 times, for each mycobacterial species. The control used corresponded to the Sauton-agar medium without of *Arbutus* extracts. The ethanol extract part II does not dissolves in the liquid culture medium sauton and thus part of ethanol extract does not test by Agar dilution assay.

(ii) Agar diffusion assay: the extracellular antimycobacterial activity of ethanol extract part I and part II was also tested by method of discs (Bauer et al., 1966). Sterilized discs of Whatman paper (diameter: 6 mm) were deposited on agar culture medium beforehand inoculated independently by 100 µl of each mycobacterial culture (approximately 10⁶ UFC/ml). The discs were deposited on 6 Petri dishes (1 disc per Petri dishes). Three discs were then impregnated with ethanol extract part I and deposited at a

dose of 160 mg of dry matter per disc on 3 other discs ethanol extract part II dried. This dose of dry matter give 6.38 mg of ethanolic extract residue part I, and 4.66 mg of ethanolic extract residue part II. The ethanol extracts residues were prepared like describe above. The Petri prepared dishes were incubated at 37°C during 3 to 4 days for rapidly growing mycobacteria and after 21 to 30 days of incubation for *M. bovis* PPI. The control used corresponded to discs impregnated with a liquid medium.

(iii) Broth dilution assay: The MIC determination of ethanol extract part I for the mycobacterial species was realized according to the broth dilution method as described by Rastogi et al. (1991). Tubes containing 6 ml of sauton liquid medium alone (control) or supplemented with increasing concentrations of ethanol extract part I, from 0.24 to 6.38 mg/ml, were inoculated with a freshly grown culture to give an OD₅₉₅ about of 0.3 for *M. bovis* PPI, and 0.2 for *M. smegmatis* MC2 and *M. aurum* A+. The tubes were then incubated with agitation at 37°C during 3 days for rapidly growing mycobacteria and 5 days for *M. bovis* PPI (Billo et al., 2005; Eldeen and Van Staden, 2008; Newton et al., 2002). The growth of control tubes has reached a D₅₉₅ of 0.4 for *M. bovis* and *M. aurum* A+, and of 0.9 for *M. smegmatis* MC₂. The MIC was determined as the minimum concentration of extract for which resulted in no visible growth in tubes (the initial absorbance of cultures has not changed). A glass marble in each tube and an agitation before the measure of the OD was necessary to avoid clumping of bacilli. This experiment was repeated four times in separate days.

Intracellular antimycobacterial activity of ethanol plant extract part I

The extracellular antimycobacterial activity of ethanol plant extract part I was tested against *M. smegmatis* inside the mice peritoneal macrophages according to the following stapes:

(i) Isolation of peritoneal rat macrophages: Peritoneal macrophage isolation was performed relying on Morissette et al. (1996) and Sosunov et al. (2007) works. Rat was injected intraperitoneally with cold Hanks balanced salt solution containing kanamycin (60 µg/ml). After 10 min, a short abdominal incision is made and the cell suspension is collected by suction to the syringe. Cells were washed twice, re-suspended in RPMI-1640 cell culture medium and 5 × 10⁵ cells/well were allowed to adhere to the bottom of 96-well tissue culture plate following an incubation period of 2 h at 37°C and 5% CO₂. The cellular density of viable macrophages was determined by Trypan Blue exclusion (Denis et al., 1990).

(ii) Infection of macrophages by bacilli: The adhered macrophages in each well of the tissue culture plate were inoculated with about 7 × 10⁵ bacilli of *M. smegmatis* MC₂. For this purpose, the medium from each well was replaced with another fresh medium containing the required bacterial concentration. Phagocytosis was allowed for 4 h at 37°C and 5% CO₂ (Rastogi et al., 1987). After phagocytosis, the medium from each well was retired and the cells were then treated during 1 h by a medium containing 10 µg/ml of amikacin. This treatment was followed by several changes of hanks balanced salt solution to remove all extracellular *M. smegmatis* MC₂ (Rastogi et al., 1987, 1991; Sharbati-Tehrani et al., 2005).

(iii) Treatment by plant ethanol extract: About 5 h after infection, necessary time of phagocytosis and elimination of extracellular *M. smegmatis* MC₂, the ethanol leaves plant extract (6.02 ± 0.76 mg/ml) which was solubilized in RPMI-1640 as demonstrated above, was added to the wells containing infected macrophages. The tissue culture plate like this prepared was then incubated at 37°C and 5% CO₂.

(iv) Enumeration of phagocytosed bacilli: After 4, 24, 48 and 72 h of treatment, the infected macrophages were lysed by adding 0.25% (w/v) sodium dodecyl sulfate (SDS), followed by a quick shaking

and then the lysates were immediately removed from each well of tissue culture plate and centrifuged. After that, SDS was eliminated; bacilli were re-suspended in Sauton medium and then were plated on the Sauton agar medium. The colony-forming units (CFU) were enumerated after about 4 days of the Petri dishes incubation at 37°C. As described by Rastogi et al. (1991), this treatment with 0.25% (w/v) SDS did not affect the viability of mycobacteria. The results were compared with the growth of the bacilli in the control culture (untreated macrophages). Results are means of 3 experiments.

(v) Cytotoxicity assay: The cytotoxicity of the peritoneal macrophages treated with the ethanol extract part I was measured by the neutral red uptake assay after 3 days of incubation (Lindl and Bauer, 1989). Only living cells are able to manage the active uptake of neutral red (Borenfreund and Puerner, 1984.). Following 3 days exposure to the ethanol arbutus extract part I (6.02 ± 0.76 mg/ml), the peritoneal macrophages that stuck to the tissue culture plate were washed with Hanks and incubated for 2 h with neutral red dye (100 µg/ml) dissolved in RPMI-1640 medium. Cells were washed again with Hanks to remove all extracellular Neutral red (Fotakis and Timbrell, 2006). The addition 10% acetic acid plus 40% ethanol solution followed by gentle shaking allowed extraction the dye from the cells in addition to its solubilization. The absorbance was read at 550 nm (Bonatto et al., 2004). The Results are compared with those of control cultures: The rat peritoneal macrophages incubated in RPMI-1640 cell culture medium and those treated with 0.25% SDS as a cytotoxic solution. The results are also means of 3 experiments.

Statistical analysis

Results were expressed as Mean ± SEM. The comparison between control and the Tread samples was analyzed using Student's t-test and p < 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Extracellular antimycobacterial activities of aqueous and ethanol plant extracts

The aqueous and ethanol *arbutus* leaves extracts part I, which follow the experimental protocol described previously, which showed a remarkable extracellular antimycobacterial activity. Indeed, the culture medium agar incorporated infusion and decoction, and ethanol plant extract part I inhibited complete growth of mycobacterial strains tested (Table 1). So we can suppose those extracts able to inhibit the proliferation of mycobacteria through one or more active substance(s). The decoction of the antimycobacterial activity observed for 15 and 30 min after decoction showed that the active ingredient responsible was not altered by heating to 100°C (Table 1). This means that the active ingredient soluble in water and ethanol is probably not such a peptid or protein. The pH of the extracts tested were adjusted to near 7, witch prove that the inhibition is due to the plant extracts studied and not to pH.

The antimycobacterial activity of the *Arbutus unedo* ethanol-extract part I is also manifested by the appearance of a large inhibition zones around the discs of Whatman paper impregnated with this extract (Table 2). These zones correspond to the inhibition of the

Table 1. Antimycobacterial effect of *Arbutus unedo* extracts.

Mycobacteria	Antimycobacterial effect of several extracts				Control
	Ethanolic extract part I	Infused	Decocted		
			15 min	30 min	
<i>M. smegmatis</i> MC ₂	-	-	-	-	+
<i>M. aurum</i> A+	-	-	-	-	+
<i>M. bovis</i> PPI	-	-	-	-	+

: Total inhibition of growth. + : Growth. The Control correspond to the cultures without extracts.

Table 2. Antimycobacterial activity of *Arbutus unedo* ethanollic extract.

Mycobacteria	Diameter of inhibition zone ¹ (mm) of ethanollic extract part I	Diameter of inhibition zone ² (mm) of ethanollic extract part II	CMI (mg/ml)
<i>M. smegmatis</i>	33.7 ± 1.86	0	6.02 ± 0.76
<i>M. aurum</i> A+	34.3 ± 0.67	0	5.59 ± 0.69
<i>M. bovis</i> PPI	34.7 ± 1.76	0	6.02 ± 0.76

¹The dose is 6.38 mg of residue / disc, corresponding to 160 mg of dry matter / disc. ²The dose is 4.66 mg of residue/ disc, corresponding to 160 mg of dry matter / disc.

bacterial growth, following the agar diffusion of the active metabolite produced. On the contrary, no zone of inhibition has been appeared around the deposit of the ethanollic extract part II. This part of the extract, used in a dry state, showed no antimycobacterial activity against the three strains tested, although it diffuses into the sauton culture medium agar.

The ethanol extract part I dose was used in this study is 6.38 mg, corresponding to 160 mg of dry matter. However, the activity detected at the same dose was similar in comparison with the obtained one against the three mycobacterial strains tested (*M. smegmatis* and *M. aurum* A+ and *M. bovis* PPI). There is no significant difference between the diameters of inhibitin zones of those mycobacteria ($p > 0.05$). These strains have the same sensitivity to the ethanollic extract part I. Dulger and Gonuz (2004) have also tested the antimycobacterial effect of ethanol extracts of 16 plants against *M. smegmatis* by the agar diffusion assay and they observe inhibition of growth by 9 species: *Nigella sativa*, *Lythrum salicaria*, *Pistacia terebinthus*, *Malva sylvestris*, *Myrtus communis*, *Origanum vulgare*, *Rosmarinus officinalis*, *Tanacetum vulgare* and *Tussilago farfara*. The zones of inhibition of extracts from these plants are lower than those obtained in this study, despite a higher dose of extract, which underscores the importance of the antimycobacterial effect *in vitro* of ethanol extract part I of *Arbutus unedo* leaves.

After having revealed the inhibitory activity of *Arbutus unedo* leaves ethanol extract part I by previous methods, MICs for the mycobacterial species studied are then determined. The MIC was 5.59 ± 0.69 mg/ml for *M. aurum* A + and 6.02 ± 0.76 mg/ ml for *M. smegmatis* MC₂

and *M. bovis* PPI (Table 2). Confirming the results obtained with the method of discs, the MIC study showed that the ethanol extract part I has approximately the same inhibitory effect against growth of the three bacterial species tested: *M. smegmatis*, *M. aurum* A+ and *M. bovis* PPI ($p > 0.05$). These results confirmed the results obtained with the method of discs.

Intracellular antimycobacterial activity of ethanollic plant extract part I

Pathogenic mycobacterial strains are intracellular parasites. Therefore, an antimycobacterial agent must be active inside cells (Draper, 1981; Mangalindan et al., 2000; Skinner et al., 1995). To visualize the intracellular Antimycobacterial activity of *Arbutus unedo* leaves ethanollic extract part I, we followed the *M. smegmatis* MC₂ growth inside rat peritoneal macrophages treated and untreated with the extract. For this way we enumerated bacillis witch were phagocytosed by the peritoneal macrophages. This enumeration consists of bacterial counts containing in the lysis solution spread on the medium culture Sauton agar.

Five hours after phagocytosis of *M. smegmatis* and the elimination of extracellular bacteria, phagocytes infected untreated and treated with ethanol extract part I were re-incubated. This time point is referred to as 0 hof post-infection. Figure 1 shows that after 4 h of post infection, the UFC of *M. smegmatis* MC₂ phagocytosed by macrophages untreated, changed from 1500 ± 50 to 1312 ± 141 UFC/well. This showed that the strain persist within phagocytes during the initial hours of post- infection.

Figure 1. Antimycobacterial effect of *Arbutus unedo* ethanolic extract against *M. smegmatis* MC₂ infecting rats peritoneal macrophages. ♦: number of intracellular surviving strains of *M. smegmatis* MC₂, enumerated in the lysate of rat peritoneal macrophages inoculated with the mycobacteria and untreated with the extract ▲: number of intracellular surviving strains of *M. smegmatis* MC₂ enumerated in the lysate of rat peritoneal macrophages inoculated with mycobacteria and treated with the extract. Repetitions number (n) between 6 and 10, ***p < 0.001. 5 h after phagocytosis of *M. smegmatis* MC₂ and the elimination of extracellular bacteria is referred as 0 h of post-infection.

Afterward, this value is gradually decreasing with the incubation period and reached 701 ± 52 UFC/well after 24 h, 387 ± 11 UFC/well after 48 h and finally 287 ± 14 UFC/well after 72 h. These results showed that *M. smegmatis* is unable to multiply inside macrophages, as reported in several previous works (Douglas et al., 2004; Sharbati-Tehrani et al., 2005). For the macrophages treated with the extract (Figure 1), we observed a significant decrease in the number of mycobacteria compared with mycobacterial load in untreated rat peritoneal macrophages. Indeed, after 4 h of post-infection, the UFC of bacilli in macrophages treated decrease immediately to 1500 ± 50 UFC/well and reach 12 ± 1 UFC/well ($p < 0.001$), and then 0 UFC/well after 24 h of post-infection ($p < 0.001$). It appears that the *Arbutus unedo* ethanolic extract part I, used at 6.02 ± 0.76 mg/ml, showed potent intracellular antimycobacterial activity against *M. smegmatis* MC₂. The extract has inactivated the majority of phagocytosed bacilli after 4 h of treatment and all intracellular bacilli after 24 h of treatment. This allowed us to conclude that the active plant constituent has a bactericidal effect on the mycobacteria located within the rat peritoneal macrophages. As *M. smegmatis* and *M.*

tuberculosis have the same sensitivity to anti-tuberculous drugs (Mitscher and Baker, 1998), we suggest that the extract of this plant would have an inhibiting effect on *M. tuberculosis* infected macrophages.

Previous phytochemical studies of *Arbutus unedo* indicate that leaves contain several phenolic compounds like tannins, flavonoids, phenolic glycosides, etc.. The strawberry leaves also contain lipids and vitamin E (Chevolleau et al., 1992; Fiorentino et al., 2007; Kivçak and Mert, 2001; Pabuçcuoğlu et al., 2003).

The antimycobacterial activity of this plant could be attributed to phenolic compounds. Accordingly, several studies have shown that the phenolic compounds isolated from a number of plant extracts have significant antimycobacterial activity (Koyasomboon et al., 2006; Mativandlala et al., 2008; Okunade et al., 2004). However, it is not excluded that other classes of fractions would be also implied. Further experiments are needed for isolation of active fractions and identification of the active components of ethanol extract.

The study of the cytotoxicity of rat peritoneal macrophages treated with the extract is performed by testing their ability to uptake neutral red. Figure 2 shows

Figure 2. Cytotoxicity of *Arbutus unedo* leaves ethanol extract (part I). I: OD 595 of the neutral red dye uptake by rat peritoneal macrophages treated with 0.25% SDS (repetitions nombre (n) = 8); II: OD 595 of the dye uptake by rat peritoneal macrophages treated 3 days by ethanol extract part I (n = 6); III: OD 595 of the dye uptake by rat peritoneal macrophages incubated in RPMI -1640 cell culture medium (n = 7). ***p < 0.001 compared with mycobacterial load in rat peritoneal macrophages treated with 0.25% SDS.

that there is no significant difference between the concentration of neutral red incorporated by peritoneal macrophages treated with the *Arbutus unedo* leaves ethanol extract part I (OD 595 = 0.5 ± 0.05) and untreated macrophages [macrophages incubated with RPMI-1640 cell culture medium (OD 595 = 0.4 ± 0.06)] $p > 0.05$. Whereas, the concentration of neutral red uptaken by macrophages treated with the cytotoxic solution of SDS is significantly lower (OD = 0.05 ± 0.008)***p < 0.001. We find that the peritoneal macrophages treated with the extract are able to uptake the red neutral dye after 3 days of treatment. This showed that the extract used at 6.02 ± 0.76 mg/ml had no toxic effect.

Conclusion

The water extract and soluble ethanol extract of *Arbutus unedo* leaves can be a promising antimycobacterial agent in the future, especially because the ethanolic extract part I shows an intracellular antimycobacterial activity and has no toxic effect on macrophages. This antimycobacterial activity could be attributed to a number of phytochemical compounds present in the aqueous and ethanolic extracts such as phenols.

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