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Original article

Antihaemolytic activity of thirty herbal extracts in mouse red blood cells

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Reactive oxygen species (ROS) can lead to haemolysis and eventually to diseases such as thalassemia and sickle cell anaemia. Their action can be counteracted by the antihaemolytic activity of therapeutic agents. The aim of our study was to identify plants that most efficiently counteract ROS-caused haemolysis. From ten plants known for their antioxidant activity (*Orobanche orientalis* G. Beck, *Cucumis melo* L., *Albizzia julibrissin* Durazz, *Galium verum* L., *Scutellaria tournefortii* Benth, *Crocus caspius* Fischer & Meyer, *Sambucus ebulus* L., *Danae racemosa* L., *Rubus fruticsos* L., and *Artemisia absinthium* L.) we prepared 30 extracts using three extraction methods (percolation, Soxhlet, and ultrasound-assisted extraction) to see whether the extraction method affects antihaemolytic efficiency, and one extraction method (polyphenol extraction) to see how much of this action is phenol-related. Extract antihaemolytic activity was determined in mice red blood cells and compared to that of vitamin C as a known antioxidant. Nine of our extracts were more potent than vitamin C, of which *G. verum* (aerial parts/percolation) and *S. tournefortii* (aerial parts/polyphenol) extracts were the most potent, with an IC_{50} of 1.32 and 2.08 μg mL^{-1}, respectively. Haemolysis inhibition depended on extract concentration and the method of extraction. These plants could provide accessible sources of natural antioxidants to the pharmaceutical industry.

KEY WORDS: Galium verum*; hydrogen peroxide; percolation; phenols;* Scutellaria tournefortii*; Soxhlet; ultrasound-assisted extraction*

Oxidative damage and haemolysis caused by reactive oxygen species (ROS) have a major role in the development of diseases such as thalassemia, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anaemia. Red blood cells (RBCs) are the primary targets of free radicals, owing to their high membrane concentrations of polyunsaturated fatty acids (linoleic and arachidonic acids in particular) and O_2 transport associated with redox active haemoglobin molecules, which are potent promoters of ROS. Oxidation depletes membrane protein content, deforms RBCs, and disturbs microcirculation (1-4). It is also implicated in haemolysis (5).

Haemolysis has long been used to measure free radical damage and counteraction by antioxidants. It is useful for screening for oxidising or antioxidising agents (6). Several herbal secondary metabolites such as flavonoids have been found to protect cells from oxidative damage. These compounds have been evidenced to stabilise RBC membrane by scavenging free radicals and reducing lipid peroxidation (3, 7).

Herbs are a rich source of flavonoids, phenolic acids, and alkaloids, some of which act as antioxidants (7-10). Nabavi et al. (9) studied the antioxidant and antihaemolytic activities of *Ferula foetida* in RBCs and a few other interesting studies have recently been reported, indicating the protective effects of plant

extracts against oxidative damage in intact RBC membranes (7, 9, 10).

The aim of this study was to investigate the antihaemolytic activity of herbal extracts from ten plants and identify promising alternatives to treating diseases associated with haemolysis. We also wanted to see which of the three extraction methods used - ultrasound-assisted extraction (UAE), Soxhlet extraction (SO), and percolation (PE) yielded more efficient extracts. Our third aim was to verify the protective effects (as claimed in literature, cf. 11, 12) of polyphenols extracted from two plants against oxidative stress. With these aims, we prepared 30 herbal extracts and evaluated their capacity to suppress RBC haemolysis. In addition, we also measured the haemolytic activity of the most potent extracts in the absence of H_2O_2 in order to determine their own oxidative toxicity to RBCs.

MATERIALS AND METHODS

Chemicals

Formic acid, 30 % hydrogen peroxide, and vitamin C, which was used as reference compound, were purchased from Merck (Darmstadt, Germany). All solvents were of analytical grade or purer. Distilled deionised water was prepared with the Ultrapure™ water purification system. Vitamin C was used in the same concentrations as the plant extracts.

Plants

Ten plant species were collected and their identity verified by Dr Bahman Eslami (Islamic Azad University of Qaemshahr, Iran). The voucher specimens were deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, Mazandaran University of Medical Sciences. Table 1 lists the plants, the parts, and the methods used for extraction. Plant materials were dried under dark conditions at room temperature for 2-3 weeks. The dry materials were milled, obtaining 2-3 mm particles.

Extraction methods

Extraction is the first important step in the recovery and purification of active ingredients from plants. The aim is to provide maximum yield and the highest quality of target compounds in the shortest time and at the lowest cost possible. Many techniques, such as conventional solvent extraction, microwave-assisted, and ultrasound-assisted extraction, have been developed to extract active ingredients (13). In this study, we opted for the three most common extraction methods: percolation, Soxhlet extraction, and ultrasound-assisted extraction.

For percolation, we used methanol to treat known amounts of each plant part at room temperature as described elsewhere (14, 15). The resulting extracts were concentrated over a rotary vacuum (Heidolph, Schwabach, Germany) at 35-40 °C until a crude solid extract was obtained, which was then freeze-dried (MPS-55 freeze-drier, Operon Co., Ltd., Gimpo, South Korea) for complete solvent removal.

In Soxhlet extraction, powdered samples were treated with methanol and extracted in an ISOLAB extractor (Wertheim, Germany) for 24 h. The extracts were then concentrated in a rotary evaporator (Heidolph, Germany) until the solvent was removed. The extracts were freeze-dried for complete solvent removal (14, 15).

In ultrasound-assisted extraction samples were treated with methanol and placed in an ultrasonic cleaning bath (Tecna3, Bologna, Italy) at a frequency of 100 kHz and temperature of 25±3 °C for 1 h to yield extracts, which were then separated from the residue by filtration and concentrated in a rotary evaporator until crude solid extracts were obtained. Followed freeze-drying for complete solvent removal (16).

Polyphenol extraction

Literature suggests that polyphenols act as antioxidants and protect RBCs against oxidative damage (11, 12, 17). These compounds were extracted from samples according to our recently published paper (16). The extraction was performed twice at 20 °C in a shaking incubator (115 W, Promax 1020, Heidolph, Germany). The extraction time was 30 min and the extracting solvent was 100 mL of methanol/acetone/water (3.5/3.5/3) containing 1 % formic acid. Extracts were combined and filtered through two layers of cheesecloth. The collected filtrate was centrifuged at 7000 *g* for 15 min. The supernatant was collected and evaporated under vacuum at 35-40 °C to remove methanol and acetone. Lipophilic pigments were then eliminated from the aqueous phase by two successive extractions in a separatory funnel with a twofold volume of petroleum ether. The aqueous phase was collected and further extracted by the equal volume of ethyl acetate three times in the separatory funnel. Three ethyl acetate phases were collected and