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Effects of Ephedrine and *Ephedra fragilis* Crude Extracts on Human Peripheral Lymphocytes

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ABSTRACT
Ephedra fragilis Desf. (*Ephedra*ceae), a locally cultivated medicinal plant, is a source of ephedrine and pseudoephedrine, two important alkaloids, which have long played an important pharmacological role. The present study investigated the *in vitro* effects of ephedrine and the *Ephedra* branch extract on unstimulated lymphocytes. *Ephedra* alkaloids were extracted from various plant parts and after phytochemical analysis, the brine shrimp lethality test was used to determine the activity of the extracts. The LC_{50} of the branch extract was 581.395 µg/ml, showing no statistical difference from that of ephedrine (208.203 µg/ml). The ephedrine and extract did not show toxic effects on lymphocytes but exhibited immunostimulant activity. Although ephedrine cannot be used as an immune booster, it can be used as a lead drug for further immunological research.
KEYWORDS: *Ephedra fragilis,* ephedrine, lymphocyte activation, cell proliferation, cytotoxicity

INTRODUCTION

Ephedra fragilis Desf. (Gnetopsida, Mormon tea, Ma Huang) is a native of the Asian countries and possesses several medicinal virtues. These include alleviation of sweating, lung and bronchial constriction, alleviation of water retention, and in the treatment of inflammation, headache, and fever (1, 2). This plant contains alkaloids (3), amino acids, and proteins (4), tannins, fatty acids (5), volatile oil, flavonoids, minerals, and vitamins. Amongst the alkaloid group, ephedrine is one of the principle alkaloids found in this plant species (3).

Clinically, Ephedrine has bronchodilator and vasoconstrictor effects, which are beneficial in the control of asthma and nasal congestion. It has diuretic properties, enhances uterine contractions, and has anti-inflammatory effects. In combination with caffeine, it has thermogenic effects, which are important for the treatment of obesity (6). However, its use has been associated with several side effects such as tachycardia, hypertension, headache, insomnia, anxiety, palpitations, dizziness, and toxic psychoses (7).

Few studies show the *in vitro* and *in vivo* immunomodulatory activity of alkaloids and related compounds. Earlier studies (8, 9) have shown that Amaryllidaceae alkaloids, ambelline and 1,2-ß-epoxyambelline, possess immunostimulatory activity. Colchicine, a phenylakylamine alkaloid, possesses immunostimulatory activity at very low doses (10). The immunostimulant compound levamisole (11, 12) is not an alkaloid but its activity has been associated with endogenous opiate alkaloids which are implied in immunostimulatory activities (13). Other phytochemical classes have been investigated in greater depth in this respect (14-17).We report here that other phenylalkylamine alkaloids (including ephedrine) possess a stimulatory rather than a cytotoxic effect on peripheral lymphocytes.

MATERIALS AND METHODS

Different aerial parts (Table 1) were collected in May 2003, from local cultivated *Ephedra fragilis* specimen on the University grounds. The plant was identified by the botanist, at the Institute of Agriculture, University of Malta where a voucher specimen is deposited. The aerial parts were separated as branches, dried pods,

seeds, fresh pods including seeds, and flowers. The material was then oven dried at 30ºC for 48 h, pulverized and dispersed in distilled water for 25 min. Each plant material was stirred for 30 min at 30ºC, allowed to stand for 15 min and stirred for another 30 min. A filtrate was obtained under reduced pressure and then treated with sodium carbonate (15 g). The solution was stirred for a further 15 min and then mixed with an equal volume of benzene. The benzene solution was then treated with acidified distilled water (2%, HCl) and the aqueous extract was neutralised to a pH of 7. The precipitate was obtained by centrifugation (Hermle, Germany) and oven-dried at 32ºC.

The dried extracts were then analysed qualitatively and quantitatively with thin-layer chromatography (Silica gel $60F_{254}$; water, acetic acid and butanol [5: 1: 4]) and UV spectrophotometry (Pharmacia LKB-Ultraspec Plus) employing standard ephedrine (Sigma) at a reference wavelength of 317 nm (Table 1). The primary bioactivity of the extracts was monitored by the brine shrimp lethality test (BST) (18). The median lethal concentrations (LC_{50}) of the respective treatments were obtained.

Human peripheral blood lymphocytes were isolated from heparinised peripheral blood of healthy human male volunteers using Histopaque®-1077 (Sigma, USA). The cells were cultured in RPMI 1640 medium supplemented with 15% foetal calf serum, antibiotics and free from PHA (a mitogen known to stimulate cell division of T-lymphocytes) at a concentration of 2.4 x 10^6 cells/ml and distributed in 96-well plates as stated below. The cultures were treated with: (a) ephedrine standard to final concentrations ranging from (69–0.69 µg/ml); (b) PHA, m-form (Gibco BRL, UK) to final concentrations ranging from 1–0.01%; (c) *Ephedra* branch extract to final concentrations ranging from 69–0.69 µg/ml (ephedrine content).

The cultures were assayed, at the specified time intervals and in triplicates, for total and viable cell counts, drug cytotoxicity and morphological characteristics. Total and viable cell counts were conducted according to the methods described by Freshney (19). Cytotoxicity was estimated using the tetrazolium LDH cytotoxicity assay (Boehringer-Mannheim, Germany) and spectrophotometric measurement of dye absorbance obtained at 492/650 nm in an ELISA reader (20). Cell suspensions treated with 100 µl Triton X-100 were used as 'high' controls, while the untreated cultures were used as 'low' controls. Cytotoxicity was estimated at 24, 48, 72, and 96 h. Morphological characteristics were observed in lymphocytes stained with Eosin Azure 50 (EA 50) as adopted from the Papanicolau method (21).

Numerical data was analyzed using the BMDP/DYNAMIC (v 7.0) (Cork, Ireland) statistical package for one-way analysis of variance (ANOVA), the Bonferroni post-hoc test for comparison of means with the control, one-way analysis of co-variance (ANCOVA) and two-tailed adjusted means T-test. Differences were considered statistically significant at a P value <0.05.

RESULTS AND DISCISSION

The different aerial parts of *E. fragilis* yielded different quantities of *Ephedra* alkaloids as illustrate in Table 1. Although branches yielded the lowest percentage of alkaloids (0.0547%), compared to the other aerial parts (> 0.1389%, $p<0.05$), the branch extract was used due a higher material availability. Asian *Ephedra* contains between 0.5 and 2.5% (6). This is slightly higher than that obtained with the locally cultivated species. In the brine shrimp test, the *Ephedra* branch extract showed a low LC_{50} (Table 2), comparable to that of Ephedrine (581.395 and 208.203 µg/ml, respectively, $p>0.05$). The fact that the LC_{50} is below 1000 µg/ml (22) indicates that the *Ephedra* branch extract exhibited activity.

The total counts over the 96 h culturing period (Figure 1) show that ephedrine and the *Ephedra* extract resulted in an increase in cell population as compared to the control. As a matter of fact the viability of the three treatments was very high over the 96-hour period (96.945 ± 0.477, 97.445 ± 0.279, and 97.621 ± 0.217%, for PHA, Ephedrine and *Ephedra* extract respectively). The control showed a slightly higher viability (97.726 ± 0.368%) although not significantly different from the rest. In many instances, the unstimulated cells are protected from apoptosis and so there is the possibility that cells remain viable without being affected by this programmed-cell death (23-25). However, proliferation tends to occur even when no stimuli are present. This was observed in this investigation as even the untreated control showed an exponential increase in total counts. The low release of lactate dehydrogenase enzyme from treated and control cells correlate well with the viability results (Table 2). This reflects a considerable consistency between the two assays carried out. Morphological results (Figure 2) reveal that for cells treated with PHA, Ephedrine and *Ephedra* extract, no qualitative differences and no evidence of apoptosis was observed. The control cells measured 6-10 µm (26), while treated cells measured 20-40 µm.

***Table 1* : The percentage extract and alkaloidal content of different Ephedra fragilis plant parts.**

Plant Part	% extract in plant material (%)[§]	Alkaloidal Content (%)[§]	Main Alkaloids
Branches	0.2723*[†]	0.0547*	E
Dried pods	0.1546*[‡]	0.1389*[‡]	E, PE
Seeds	0.6711	0.6234	PE
Pods/seeds	0.5894	0.5198	E
Flowers	2.0780*‡	1.8675*‡	E, PE

[§]Values expressed as % in dried plant material

*p<0.05; [†] denotes lower yield and [‡] denotes higher yield than the other extracts. E = ephedrine, PE = pseudoephedrine

Table 2 : The median lethal concentrations and cytotoxic activities for the extracts and PHA, for the brine shrimp test (BST), viability and cytotoxicity assay (LDH) at 48 h, respectively.

Solvent/Plant Combination	LC_{50}, µg/ml (BST)	Viability (%)	LDH (%)
PHA	-	96.945±0.477	3.780±0.243
Ephedrine	208.203	97.445±0.279	3.821±0.353
Ephedra extract	581.395	97.621±0.217	4.098±0.211

Values expressed as means ± S.E.M.

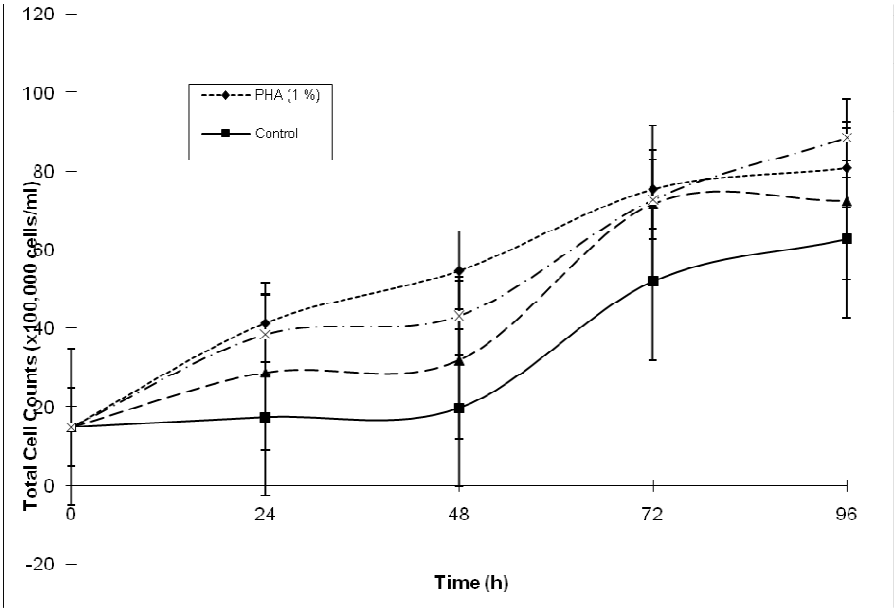

Figure 1 : Total Counts over the 96 h culturing time for untreated lymphocytes and those treated with PHA (1%), Ephedrine (69 µg/ml) and Ephedra extract (69 µg/ml). Data shown were the mean ± SEM of ten determinations.

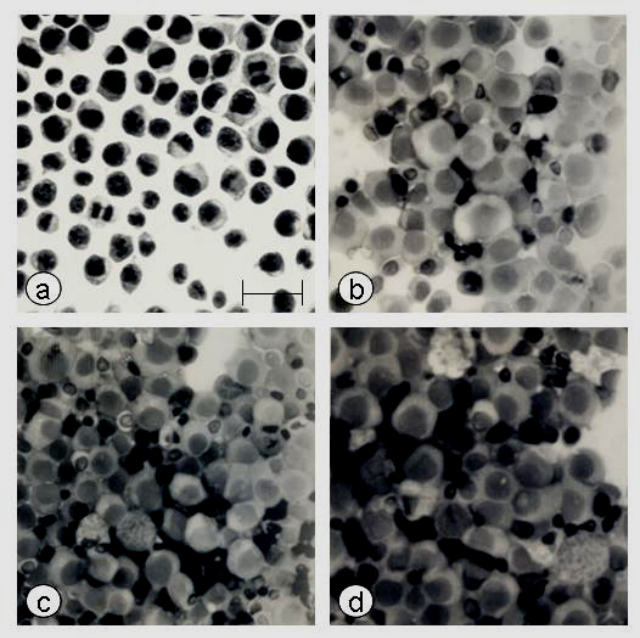

Figure 2 : Morphological characteristics for lymphocytes (a) left untreated, and treated with (b) 1 % PHA, (c) 69 µg/ml ephedrine and (d) 69 µg/ml Ephedra extract (Scale 20 µm).

Ephedrine, like the catecholamines, has both direct and indirect adrenergic activity (27). However, catecholamines have a selected affinity towards one type of adrenergic receptor. For example, phenylephrine has a preferential α-adrenergic activity while norepinephrine and epinephrine possess higher ß-adrenergic activity affinity. *In vitro*, ß-adrenergic agonists tend to inhibit stimulated cells, including lymphocytes, while α-adrenergic agonists do not inhibit this activity (28). However, ß-agonists have been shown to exhibit a stimulatory response on lymphocytes only in an *in vivo* system, involving a spleen-dependent process (29). Ephedrine differs from ephinephrine and phenylephrine as it lacks the two catechol hydroxyl groups which are responsible for α and ß-adrenoceptor affinity. The N-substituted moiety and the ß-hydroxyl moiety are particularly important for ß-adrenergic activity (30). A distinguishing characteristic is that ephedrine has more activity at α-adrenoceptors rather than pseudoephedrine, and the opposite towards ß-adrenoceptors. This suggests that the stereo-arrangement of the ß-hydroxyl moiety is critical. As a matter of fact it can be concluded that owing to the fact that ephedrine is the most predominant alkaloid in the *Ephedra* branch extract and direct lymphocyte activation *in vitro* is mainly due to α-adrenoceptor activity, it leads to the conclusion that the *Ephedra* branch extract, more particularly ephedrine, exhibit a potent direct effect on lymphocytes *in vitro*. In spite of this, the stimulation of a T-lymphocyte subset could be of importance in tumour immunology (31-33). This has been suggested for other plant metabolites, such as Amaryllidaceae alkaloids, extracted from *Crinum latifolium* L. (8, 9) and Cucurbitaceae terpenoids, extracted from *Ecballium elaterium* (L.) A.Rich (Cucurbitaceae) (34). Although *in vivo*, *Ephedra* (Ma Huang) has shown to cause mild myocyte hypertrophy and infiltration with lymphocytes, associated with ephedrine (35), its main constituent may be used as a lead compound for further immunomodulatory research.

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