Argania spinosa is an evergreen tree endemic of southwestern Morocco. Many preparations have been used in traditional Moroccan medicine for centuries to treat several illnesses including diabetes. However, scientific evidence supporting these actions is lacking. Therefore, we prepared various extracts of the argan fruit, namely keel, cake and argan oil extracts, which we tested in the HTC hepatoma cell line for their potential to affect cellular insulin responses. Cell viability was measured by Trypan Blue exclusion and the response to insulin evaluated by the activation of the extracellular regulated kinase (ERK1/2), ERK kinase (MEK1/2) and protein kinase B (PKB/Akt) signaling components. None of the extracts demonstrated significant cytotoxic activity. Certain extracts demonstrated a bi-phasic effect on ERK1/2 activation; low doses of the extract slightly increased ERK1/2 activation in response to insulin, whereas higher doses completely abolished the response. In contrast, none of the extracts had any significant effect on MEK whereas only a cake saponin subfraction enhanced insulin-induced PKB/Akt activation. The specific action of argan oil extracts on ERK1/2 activation made us consider an anti-proliferative action. We have thus tested other transformed cell lines (HT-1080 and MSV-MDCK-INV cells) and found similar results. Inhibition of ERK1/2 activation was also associated with decreased DNA synthesis as evidenced by [3H]thymidine incorporation experiments. These results suggest that the products of Argania spinosa may provide a new therapeutic avenue against proliferative diseases.

Keywords: cancer – diabetes – MAPK – PKB – transformed cell lines

Introduction

The argan tree (Argania spinosa) is a thorny evergreen tree that plays an essential ecological and socio-economic role in southwestern parts of Morocco. A program aimed to increase the industrial value of Argania spinosa is currently carried out in Morocco (1,2). All the parts of the tree have value. Specially, the almond oil is largely used for cooking or in traditional medicine and represents the argan tree’s most important product.

Various biological properties largely known by traditional medicine have received some scientific supports. It is notably the case for the traditional uses of almond oil to decrease blood cholesterol and hypertension (3–5), to treat skin diseases such as juvenile acne and chicken pox pustules (2), or for other beneficial effects of saponin derivatives, notably analgesic and anti-inflammatory (6), anti-microbial (2) and lipolytic properties (2).

Chemical analysis revealed that argan oil is particularly well balanced in terms of fatty acid composition (2,7). Argan oil is...
Molecular action of argan seed extracts

Molecular action of argan seed extracts

Biomedicals, LLC (Irvine, CA).

Materials and Methods

Chemicals

Phospho-specific antibodies p44/42 MAPK\textsuperscript{Thr202}, PKB/Akt\textsuperscript{Ser473}, MEK1/2\textsuperscript{Ser217} and corresponding non-phospho-specific antibodies corresponding kits were purchased from Cell Signaling Technology (Beverly, MA). Bovine pancreas insulin as well as other chemicals were obtained from Sigma-Aldrich Canada (Oakville, ON). Tritiated thymidine ([methyl-\textsuperscript{3}H] TdR; 4.0 Ci/mmol) was procured from MP Biomedicals, LLC (Irvine, CA).

Argania spinosa Extracts

The Argania spinosa extracts were prepared at the Chemistry Laboratory of Plants Chemistry, Organic and Bioorganic Synthesis headed by Prof. Z. Charrouf. The argan fruit almonds, cake and oil were kindly provided by the Argan Oil Womens Cooperative (Targanine), Morocco, and prepared according to procedures previously described (8,12,13). Figure 1 summarizes the preparation of different extracts. All extraction procedures were carried out under normal lighting conditions. Almond extract A1 was obtained by macerating ground argan almonds (407 g) at room temperature in 80% methanol for 36 h with agitation. The solvent was then evaporated and the residue dried to obtain a fine whitish powder (42.4 g; 10.4% yield; Fig. 1). Almonds were also mechanically pressed at 50–60°C in order to separate argan oil and press cake. The press cake (543 g starting material) was first defatted with hexane and extracted with methanol at room temperature giving extract B1 (54.3 g; 10% yield). Additionally, two saponin fractions were obtained from the press cake following extraction with methanol as follows. The methanol extract B1 was partitioned between water and chloroform; the water soluble portion was successively extracted with ethyl acetate and n-butanol. The n-butanol extract (6.8 g) was dissolved in minimum water and chromatographed on diaon HP20 column (80 × 2 cm) sequentially with water, 30% MeOH and 100% MeOH. After evaporation of MeOH from the last fraction, the polar fraction of press cake saponins B2 (2.1 g) and a less polar fraction from the 30% methanol B3 extract (1.2 g) were obtained, the latter being collected after treating the water soluble portion with n-butanol. Finally, an unsaponifiable fraction C1 was obtained from argan oil. The oil (20 g starting material) was well mixed with 200ml of ethanol and potassium hydroxide (KOH 2N/EtOH analysis) and heated (at ~44°C) with backward flow for 1 h. Following addition of distilled water (200 ml) and cooling to room temperature, hexane was added (150 ml). This mixture was shaken vigorously for 1 h and the partitioned hexane layer was collected. The remaining aqueous phase was extracted twice more with hexane (150 ml). The hexane extracts were pooled, washed with water/ethanol (50 : 50), dehydrated over anhydrous Na\textsubscript{2}SO\textsubscript{4} and filtered. The solvent was removed using a rotavapor. The residue (1.2 g) was heated at 103°C for 1 h. Cake saponin extracts B2 and B3 were dissolved in water. The three other extracts namely A1, B1 and C1 were dissolved in DMSO. The maximal DMSO content in culture medium was set at 0.1 % (v/v).

Cell Culture

HTC cells express insulin receptors and signaling pathways similar to those found in primary rat hepatocytes (14,15). HTC cells were cultured in minimal essential medium (MEM) (Sigma, St Louis), supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under a humidified atmosphere of 5% CO\textsubscript{2}/95% air.

Materials and Methods

Chemicals

Phospho-specific antibodies p44/42 MAPK\textsuperscript{Thr202}, PKB/Akt\textsuperscript{Ser473}, MEK1/2\textsuperscript{Ser217} and corresponding non-phospho-specific antibodies corresponding kits were purchased from Cell Signaling Technology (Beverly, MA). Bovine pancreas insulin as well as other chemicals were obtained from Sigma-Aldrich Canada (Oakville, ON). Tritiated thymidine ([methyl-\textsuperscript{3}H] TdR; 4.0 Ci/mmol) was procured from MP Biomedicals, LLC (Irvine, CA).
Other cells used were the HT-1080 (human fibrosarcoma) cell line, and the invasive variant of Moloney Sarcoma Virus transformed Madin-Darby Canine Kidney cells (MSV-MDCK-INV) (16) possessing motile and invasive properties. They were cultured in Dulbecco’s minimal essential medium (DMEM) containing 10% FBS, 2 mM glutamine, 1% non-essential amino acids, 0.5% vitamins, 1% penicillin and streptomycin under 5% CO₂ atmosphere at 37°C (16,17).

**Cell Viability Studies**

Confluent cells were incubated in MEM + 0.5% FBS with different doses of extracts. Following 6 or 21 h incubation periods, cells were rinsed with calcium-free PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄ and 8.1 mM Na₂HPO₄, pH 7.4) and loosened with trypsin (0.05% trypsin and 0.53 mM EDTA) for 5 min at 37°C. Cell viability was then determined by Trypan Blue exclusion (0.2% final concentration).

**Treatments**

Confluent HTC cells were rinsed and incubated in MEM containing 0.5% FBS and extracts at different doses (12.5, 25, 50 and 100 µg ml⁻¹) for periods of 5 or 20 h. Additional 1 h incubation with fresh extracts was done in serum-free medium. This procedure ensured that intracellular signaling pathways activated by growth factors resumed baseline activity. Following treatments with extracts, insulin (0–100 nM final concentration) was added to the medium for 5 min at 37°C. The stimulation was stopped by quickly removing the medium and washing the cells twice with ice-cold phosphate-buffered saline (PBS). Cells were solubilized in lysis buffer [25 mM Tris–HCl, pH 7.4, 2 mM benzamide, 1 mM phenylmethanesulfonyl fluoride and 10 µg ml⁻¹ aprotinine] and the phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate and 10 mM sodium pyrophosphate). Lysis was carried out for 5 min on ice followed by scraping. Lysates were recovered and insoluble material was removed by centrifugation (4500 g for 10 min) at 4°C. The supernatant was kept at −80°C until analyzed.

HT-1080 and MSV-MDCK-INV cells were seeded at 1.80 × 10⁵ cells per 60 mm plate and cultured as indicated for ~24 h before use. About 18 h before the beginning of the incubation, at 60–70% confluence, the initial medium was changed for a DMEM medium containing 0.5% FBS. Cells were then incubated with serum-free medium containing *Argania spinosa* extracts (doses ranging from 0.79 to
12.5 μg ml⁻¹ as indicated) for 6 h. Cells were washed three times with PBS/CM (PBS/0.1 mM CaCl₂, 1 mM MgCl₂), and lysed with 3× Laemmlli sample buffer (15% β-mercaptoethanol, 7.5% SDS, 30% glycerol, 300 mM Tris–HCl pH 6.8 and bromophenol blue) heated at 95°C. The cell lysates were harvested and kept at −80°C pending immunoblot assays.

**Immunoblot Analysis**

Cell lysates were subjected to electrophoresis on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and blotted with phospho-specific p42/44 MAPKThr202Tyr204 (1 : 2000), PKB/AktSer473 (1/1000) or MEK1/2Ser217 (1 : 2000) antibody. Equal protein loading was routinely ascertained following transfer to nitrocellulose membranes by Ponceau Red staining. Moreover non-phosphorylated forms of the proteins were detected by following antibodies stripping and incubation of membranes with pan-specific antibodies, namely anti-Akt (1 : 1000), anti-ERK1/2 (1 : 1000) or anti-MEK1/2 (1 : 1000). The antibody-antigen complexes were detected with anti-mouse or anti-rabbit antibodies coupled with horseradish peroxidase and detected using an enhanced chemiluminescence kit (Perkin Elmer, Boston, MA).

**Anti-proliferative Activity**

HT-1080 and MSV-MDCK-INV cells were seeded into 24-well plates at a density of 1 × 10⁵ cells per well and cultured 24 h in 10% FCS supplemented medium. They were starved in serum-free medium for another 24 h and then incubated for 24 h in 10% FCS supplemented medium. They were exposed to 50 μg ml⁻¹ of each extract for 6 or 21 h and viability was then determined by Trypan Blue (0.2%) exclusion. The suitable controls (DMSO or H₂O) were carried out as indicated in the Materials and Methods section.

**Results**

**Argania spinosa Extracts are not Cytotoxic**

We first examined the effects of the different argan seed extracts on HTC viability. As shown in Table 1 the extracts do not exhibit any cytotoxic activity toward HTC cells. This was true for concentrations as high as 50 μg ml⁻¹ and for incubation periods of 6 or 21 h.

**Methanol Extracts of A. spinosa Almonds and Cake Exhibit Bi-phasic Modulation of Insulin Signaling in HTC Cells**

Next, HTC cells were treated with various argan seed extracts for 6 or 21 h and their action on the cells’ responsiveness to insulin was assessed by measuring phosphorylation of ERK1/2 and Akt/PKB. Treatment of HTC cells with the methanolic extract of argan almond (A1) or cake (B1) induced a bi-phasic effect on the response of ERK1/2 and, to lesser extent of Akt, to insulin stimulation. Indeed, short- or long-term exposure of cells to 12.5 μg ml⁻¹ of A1 (Fig. 2A and B, respectively) and B1 (Fig. 3A and B) enhanced slightly the activation of ERK1/2. However increasing A1 concentration to 25 μg ml⁻¹ completely abolished ERK1/2 response and diminished that of Akt after either 6 or 21 h incubation (Fig. 2C and D). This bi-phasic behavior is highlighted in Fig. 2E, where Akt and ERK1/2 activation is presented in relation to respective DMSO controls for both concentrations of A1 extract used over a 6 h period. Similar results were observed with B1 at 25 μg ml⁻¹ (results not illustrated).

**A. spinosa Cake Polar Saponins Selectively Enhance Insulin-dependent Akt Activation in HTC Cells**

In contrast, B2 saponin extract increased Akt phosphorylation without affecting that of ERK1/2. As illustrated in Fig. 3E, the positive effect of B2 extract on Akt phosphorylation was more pronounced after a 21 h incubation period (immunoblot in Fig. 3D) as compared with 6 h (immunoblot in Fig. 3C, see also Fig. 3E). On the other hand, B3 extract did not have any significant effect on either ERK1/2 or Akt/PKB activation by insulin, be it after 6 or 21 h incubations (Fig. 3C and D, respectively).

**The Unsaponifiable Fraction of A. spinosa Oil Extract Selectively and Potently Inhibits Insulin-dependent and Insulin-independent ERK1/2 Activation in HTC Cells**

The unsaponifiable extract of argan oil, C1, had a different pattern of action on HTC liver cells. At a concentration of 25 μg ml⁻¹, C1 treatment reduced the ability of ERK1/2 to respond to increasing doses of insulin, whereas the Akt response remained undisturbed. This effect became more prominent at a concentration of 50 μg ml⁻¹. The ERK1/2 response was abolished completely when C1 incubation time was...
increased from 6 to 21 h (Fig. 4). Cell lysates were then probed for phosphorylated MEK1/2, the kinases lying immediately upstream of ERK1/2 in this insulin-signaling cascade. As shown in Fig. 4, insulin-induced activation of MEK1/2 was not decreased by C1 extract irrespective of the dose or incubation period used. If anything, treatment with C1 extract appeared to slightly increase basal MEK1/2 phosphorylation.

The Unsaponifiable Fraction of *A. spinosa* Oil Extract Inhibits Proliferation of Several Transformed Cell Lines in a Dose-dependent Manner through ERK1/2 Inactivation

These intriguing results indicated that C1 extract could specifically interrupt the insulin-signaling cascade between MEK1/2 and ERK1/2. In view of the fact that this signaling cascade is involved in the proliferative action of insulin (also shared by growth factors), it seemed appropriate to examine the anti-proliferative effect of C1. For this purpose, we selected two cell lines of tumorigenic origin, namely the human HT-1080 fibrosarcoma cell line and the transformed and invasive canine MSV-MDCK-INV cells. As shown in Fig. 5, exposure to increasing concentrations of C1 extract inhibited DNA synthesis in both cell types in a dose-dependent manner. At a dose of 12.5 µg ml⁻¹, C1 completely abolished [³H]thymidine incorporation. When the activation states of MEK1/2 and ERK1/2 were examined, a dose of 12.5 µg ml⁻¹ of C1 strongly inhibited ERK1/2 without affecting MEK1/2 (Fig. 6). This behavior is similar to that seen previously in HTC cells (Fig. 4). Furthermore, the effect of C1 extract was comparable to that of U0126 a well-known inhibitor of MEK1/2 activity. Hence, C1 possesses strong anti-proliferative activity that appears to be mediated by the interruption of signaling cascades at the MEK1/2–ERK1/2 interface.

Discussion

It recently came to our attention through Moroccan folk medicine that argan almond may be beneficial for the treatment of diabetes (Z. Charrouf, unpublished data). This study was thus carried out to examine the potential anti-diabetic action of various fractions of the seed of the argan tree, *Argania spinosa,*
and to begin exploring their mechanism of action. Methanolic extracts of the almonds (A1), and cake (B1), were prepared as well as two fractions of cake saponins (B2 and B3) and an unsaponifiable fraction of argan oil (C1). None of the fractions exhibited any cytotoxic activity toward a model liver cell line, HTC cells.

Being an insulin-responsive cell type, HTC cells were used to test the hypothesis that some of the *Argania spinosa* seeds extract may exert some insulin-sensitizing action. We measured two key enzymes involved in mediating the physiological responses to insulin in target cells. Akt/PKB lies downstream of phosphatidylinositol-3-Kinase (PI3K) and mediates the effect of insulin to enhance glucose transport in muscle and adipose tissues (11). The enzyme ERK1/2, on the other hand, is implicated in the action of insulin on gene transcription and cell division (11). In the liver, both enzymes participate in insulin-stimulated glycogen synthesis (18). Figure 7 summarizes the results obtained in the present studies.

Firstly, total methanolic extracts of the almond (A1) and press cake (B1) had a bi-phasic (concentration-dependent) effect on ERK1/2 and Akt activation by insulin (Fig. 7). A concentration of 12.5 µg ml⁻¹ of A1 had a small stimulating action on both enzymes, whereas 25 µg ml⁻¹ strongly inhibited ERK 1/2. When a saponin-rich fraction of the pressed...
cake was extracted with 100% methanol (B2 extract), a more potent stimulatory effect on insulin-induced Akt activation was uncovered and modulation of ERK1/2 was lost. In contrast, isolating the unsaponifiable fraction from Argan oil C1 extract yielded the loss of enhanced Akt activation but was characterized with a powerful inhibitory action at the level of ERK1/2. This result along with the bi-phasic nature of the effects of cruder almond and pressed cake extracts argues for the presence of at least two active components in seeds of *Argania spinosa*.

A first component appears to be present in the B2 saponin fraction of the pressed cake and could underlie an insulin-sensitizing action that offers a possible mechanism for the alleged anti-diabetic potential of *Argania spinosa* almonds. Indeed, previous work by Alaoui et al. (19) demonstrated a hypoglycaemic effect in rats treated with 100 mg kg⁻¹ per day of an oral mixture of press cake saponins that occurred within one month. The hypoglycaemic effect could be related to enhanced transport of glucose into adipose or skeletal muscle tissues, which are known to be activated through the PI3K pathway. Few studies describe hypoglycaemic properties of the triterpenic type saponins. Yoshikawa et al. (20) have elucidated the structure of saponins present in the pressed cake of *Argania spinosa* which are present in the extracts tested herein. Using high performance liquid chromatography (HPLC) we have found seven saponins, five of which are novel molecules. All are similar to saponins used by Yoshikawa and Matsuda (20). The results of the present study suggest that such saponins enhance insulin sensitivity in an insulin-responsive cell line of hepatic origin. Taken together, this evidence supports the use of *Argania spinosa* almonds for the prevention or treatment of diabetes, albeit clinical confirmation is still required.

Higher concentrations of the methanolic extracts (A1) of almond, of the pressed cake (B1) and of the unsaponifiable fraction (C1) slightly decreased the degree of phosphorylation of Akt in HTC cells, but induced a complete inhibition of ERK1/2 phosphorylation. The C1 extract specifically reduced the activation of ERK1/2 without affecting the degree of phosphorylation of MEK1/2 on two other transformed cell lines HT-1080 and MSV-MDCK-INV cells. At high concentration, its effect is comparable to that of U0126. This MEK inhibitor does not affect the phosphorylation status of MEK1/2 but inhibits its catalytic activity by binding to activated MEK in a non-competitive manner. This prevents the enzyme from phosphorylating and activating downstream ERK1/2. It is...
conceivable that C1 may inhibit MEK activity in a similar manner. Alternatively, C1 could inhibit the phosphorylation of ERK1/2 by another mechanism, such as activation of a specific phosphatase. Further studies will be necessary to address this point. Nevertheless, these results definitively point toward an anti-proliferative activity of A1 and C1 extracts rather than an anti-diabetic one. This was confirmed for C1 by a reduction of [3H]thymidine incorporation.

Argania spinosa almonds and press cake have not yet been subjected to detailed phytochemical analysis. In contrast, some phenolic compounds have been identified in argan oil including ferrulic acid, vanillic acid, tyrosol and syringic acid, carotenoids (8), caffeic acid and oleuropein (24). The unsaponifiable fraction of argan oil is rich in lipid-soluble vitamins, phytosterols, triterpenes, carotenoids and squalene components believed to confer cancer chemopreventive potential (8,25,26).

Phenolic or flavonoid compounds had long been recognized as potent antioxidants (27,28). Antioxidants are believed to play a role in cancer prevention (29,30), although the exact mechanisms have not been elucidated. In relation to the present studies, it must be noted that some flavonoids have shown an ability to inhibit the MEK/ERK cascade in rat hepatoma 5L cells (31). More specifically, the flavonoid naringenin impaired the PI3K and MAPK pathways; it inhibits the phosphorylation of ERK1/2 without affecting the upstream kinase, MEK (27). This is similar to the effect of A1, B1 (not shown) and C1 extract observed herein. Moreover the antitumor effect of another flavonoid, nobiletin, on human fibrosarcoma HT-1080 cells (28) was associated with the inhibition of MEK1/2 activity without influencing the activity of PI3K or the phosphorylation of Akt. Finally, ferulic acid exhibits a potent anti-proliferative effect through inhibition of ERK1/2 pathway (32). It is thus possible that flavonoids contained in the almond of Argania spinosa could participate in the inhibition of ERK1/2 pathway and be involved in the observed anti-proliferative activity. We do not expect the C1 fraction to contain flavonoids but it is possible that other components of the unsaponifiable fraction may have the same mechanism of action.

Components such as lipid-soluble vitamins, specifically vitamin E, may be involved in the action of C1 extract. Indeed, argan oil is rich in tocopherols (700 mg Kg$^{-1}$) (8); principally $\gamma$-tocopherol (75%) and $\alpha$-tocopherol (7%). $\gamma$-tocopherol is known for its cancer-preventive properties (33–36). $\alpha$-tocopherol is also known to inhibit proliferation of cancer cells (37) and to interfere with signal transduction pathways such as PKC and ERK (38).

In addition, the unsaponifiable fraction of argan oil shows a particular wealth of phytosterols, principally in the form of schwottenol (48%) and spinasterol (40%), with minor amounts of $\delta$-8,22-stigmastadiene-3$\beta$-ol. These sterols are very rare in vegetable oils. Schwottenol has been reported to exhibit anticarcinogenic, cytotoxic properties (39) and strong antitumor promoter activity (40). The anti-proliferative and antitumorogenic potential of spinasterol has also been demonstrated (41–43). Finally, some triterpenes, such as lupeol contained in argan oil unsaponifiable fraction, can target multiple signaling pathways leading to induction of apoptosis and inhibition of cell growth (44,45). Even if comparable to that of several natural compounds, such as certain flavonoids, the effect of these unsaponifiable components on signaling pathway enzymes is as yet unclear. Nonetheless, phytosterols, tocopherols and triterpenes in C1 extract could all contribute to its anti-proliferative activity.

Finally, squalene is another important component of argan oil (320 mg Kg$^{-1}$) known as an excellent...
The anti-hypercholesterolemic agent through suppression of HMG-CoA reductase activity, shown to intervene in the release of oncogenic activity (46,47). Squalene has also been reported to exert anti-tumorigenic (48) or anticarcinogenic effects (47) as well as glutathione-like cytoprotective activity (49). Finally, the anti-proliferative activity of squalene appears to involve inhibition of the Ras/Raf/MEK/ERK pathway (50).

Conclusion

While many single components of Argania spinosa seeds could be responsible for the effects observed in this study, synergy or antagonism between multiple components is also likely. However, using a fractionation approach can help unravel such complexities. Indeed, we prepared several extracts from argan seeds, its oil and remaining press cake. This allowed us to demonstrate an insulin-sensitizing activity present in saponin-rich presscake fractions that lends support to the traditional use of argan almonds against diabetes. However, a bi-phasic modulation of ERK1/2 component of the insulin response by treatment with argan seed extract could be explained by anti-proliferative components concentrated in the unsaponifiable fraction of argan oil. Hence, our studies confirm in part the anti-diabetic and cancer chemopreventive potential of Argania spinosa seeds. They further suggest that active principles for each action may reside in different fractions and this may help their identification in future studies.

Moreover, natural health products of vegetable origin were clearly identified as a promising avenue for the prevention of chronic diseases (51), as raised notably by a panel of experts that convened in Montreal in 2004 (52). Our study supports the concept that A. spinosa almonds, oil, and extracts thereof
are part of such natural health products that can be useful in the prevention of serious chronic illnesses like cancer and diabetes.

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