Table 2: Antioxidant activity of *Rhamnus alaternus* bark extracts measured by means of different *in vitro* tests

Tests	*Rhamnus alaternus* bark extracts		
	Methanolic	Aqueous	Traditional
Folin µg GaE/mg extract	190.23±12.117	162.72±0.603	181.56±4.034**
DPPH mmol TE/g extract	0.61±0.108	0.39±0.050	0.54±0.077
FRAP mmol Fe^{2+}/g extract	1.72±0.068	1.240±0.052	1.26±0.056*
TEAC mmol TE/g extract	0.75±0.001	0.65±0.002	0.66±0.004*
ORAC mmol TE/g extract	6.55±0.027	5.27±0.123	3.96±0.093*

Results were expressed as mean±SD of three experiments. *Versus methanolic extract $P<0.01$, by Student's *t*-test; **Versus aqueous extract $P<0.01$, by Student's *t*-test. GaE: gallic acid equivalent; TE: Trolox equivalent; SD: Standard deviation; DPPH: 2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing/antioxidant power; TEAC: Trolox equivalents antioxidant capacity; ORAC: Oxygen radical absorbance capacity

in the materials and methods section. In normal PBMCs, the traditional extract significantly induced cell death in a dose-dependent manner so that the concentration inducing the 50% of cell death (IC_{50}) was 29.35 µg/ml [Table 3]. When the aqueous extract was tested, the concentration inducing the 50% of cell death was similar (IC_{50} 38.81 µg/ml) to that of the traditional extract, even if at less extend. Conversely, the methanolic extract produced less cell death with the highest IC_{50} (220.35 µg/ml) after 24 h.

Data analysis of trypan blue assay, as shown in Table 3, also revealed IC_{50} values of the *R. alaternus* bark extracts on human leukemic monocyte lymphoma cell line (U937) after 24 h of exposure. Proliferation was most potently suppressed by the methanolic extract with the lowest IC_{50} value (6.39 µg/ml). Proliferation of U937 cells exposed to traditional and aqueous extracts was not significantly reduced resulting in higher IC_{50} values (84.65 µg/ml and 76.74 µg/ml respectively).

The criteria of cytotoxicity for the crude extract, established by the U.S. National Cancer Institute (NCI) in the preliminary assays, report IC_{50} values of 10–20 µg/ml of a total/crude extract as cytotoxic, 20 to ≤50 µg/ml as moderately cytotoxic, and <10 µg/ml as strongly cytotoxic.[28] The IC_{50} value for the methanolic extract of *R. alaternus* bark on U937 cells was found to be lower than that specified by NCI for categorization as anticancer agent. On the contrary, the traditional and aqueous extracts can be considered as poorly cytotoxic for U937 cells. Furthermore, these data are also interesting as they suggest that the methanolic extract possesses a cytotoxic activity specifically on cancer cells (U937), while the traditional and aqueous extracts are moderately toxic on normal PBMCs.

DISCUSSION

The aim of this preliminary study was to characterize the flavonoid content, the antioxidant properties and the cytotoxic activity of different extracts obtained from Algerian *R. alaternus* bark, a drug used in North African folk medicine.

Table 3: IC_{50} values (concentration eliciting 50% inhibition) for *Rhamnus alaternus* extracts and taxol in PBMC cells and U937 cells. Cells were treated with various concentrations of the drugs, and the cell number was counted after 24 h of exposure

Rhamnus alaternu bark extracts	IC_{50}- µg/ml (confidence limits)	
	PBMCs	U937
Methanolic	220.35 (186.64-260.41)	6.39 (4.10-9.95)
Aqueous	38.81 (18.06-53.39)	76.74 (58.56-98.75)
Traditional	29.35 (17.59-53.52)	84.65 (57.39-101.17)
Taxol	19.46 (12.35-22.54)	2.47 (1.48-3.35)

PBMCs: Peripheral blood mononuclear cells; IC_{50}: 50% inhibition concentration

The three extracts under investigation show a similar flavonoid profile, but the methanolic extract has a value of flavonol content (51.17 ± 0.409 µg QE/mg) significantly higher than that of two extracts obtained by water (24.096 ± 0.852 and 12.03 ± 0.88 µg QE/mg of extract respectively). This may be due to the low water solubility of flavonoids, needing an organic solvent to be extracted from the vegetable matrix. Furthermore, the traditional extract obtained by decoction contains, despite the heating, an amount of flavonoids lower than that of the aqueous one obtained by maceration, very likely because according to the traditional methodology this extract was obtained from 10 g of raw drug, thus an amount lower than that (50 g) employed to obtain the other two extracts.

As to the antioxidant power, in all the usually classified as ET assays (DPPH, Folin, FRAP, TEAC), the three extracts show a similar free-radical scavenging power, being the methanolic extract more effective than the others. Conversely when the HAT based assay ORAC was employed, the effectiveness order was: Methanolic extract > aqueous extract > traditional extract.

It is generally considered that the primary mechanism of the radical scavenging activity of flavonoids is

hydrogen atom donation, although they may also act by single-electron transfer. Thus our findings, taken together, suggest that the antioxidant activity of *R. alaternus* bark extracts may be due, at least partially, to their content in flavonols but also it demonstrate that the aqueous extract and especially the traditional one contains a significant amount of compounds, different from flavonoids, acting by single-electron transfer, which contribute (independently on the characteristics of chemical environment) to the antioxidant activity measured in the ET-based assays. Furthermore, it is evident that, also with regard to the antioxidant power, the traditional methodology (decoction) allows to obtain an extract which (although starting from a less drug amount) retains a good efficacy, similar to that of an aqueous extract produced without heating, and very likely due to the contribution of compounds released from the plant matrix by heating.

Cytotoxicity of *R. alaternus* extracts was evaluated in a leukemic cancer cell line (U937) and in normal PBMCs. This method gives an indication of potential usefulness in a clinical setting, for which selectivity in favor of the cancer cell line being the more susceptible is required.[29] The aqueous extract and at a higher degree, the traditional one produced a marked decrease in the viability of PBMCs, with IC_{50} of 38.81 μg/ml (C.L. 18.06–53.39) and 29.35 μg/ml (C.L. 17.59–53.52), respectively. Conversely, the methanolic extract exerted only a weak cytotoxic effect on normal PBMCs, with IC_{50} of 220.35 μg/ml (C.L. 186.64–260.41), which is a clear indication that normal human cells may be more resistant to this extract.

As to the experiments on leukemia U937 cells, the methanolic extract (richer in kaempferol, rhamnocitrin, and quercetin derivatives), but not the other two extracts, decreased significantly the viability of these cells, showing an IC_{50} of 6.39 μg/ml (C.L. 4.10–9.95), with the suggested effective doses for a 50% inhibition in cell viability for plant extracts being <20 μg/ml and thus to be considered active according to the NCI guidelines.[28]

Kaempferol and rhamnocitrin glycosides from *R. alaternus* have been demonstrated to induce apoptosis in human lymphoblastoid cells TK6,[12] and quercetin can induce caspase-dependent cell death in U937 cells.[30] Thus, we can suppose that the cytotoxic effect of the methanolic extract under study is due to kaempferol, rhamnocitrin, and quercetin derivatives contained in it. As to the other two extracts, they do not contain a sufficient amount of these compounds to obtain an evident cytotoxic effect on leukemia cells and on the other hand, they contain other hydrophilic compounds toxic for normal PBMCs.

CONCLUSION

These preliminary data evidence that *R. alaternus* bark can be considered as a good source of bioactive compounds possessing significant antioxidant and cytotoxic activity, in particular kaempferol, rhamnocitrin, and quercetin derivatives. However, only the methanolic extract is able to decrease specifically the growth of cancer cells, such as the human leukemia cells U937, while the water extracts obtained by maceration or by decoction are moderately cytotoxic for normal human cells. Further studies are warranted to optimize the conditions needed to obtain from *R. alaternus* bark an extract enriched in anticancer flavonols but lacking of compounds toxic for normal cells.

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