-Confidential-

Local Food-Nutraceuticals

NEWSLETTER

EDITORIAL by Prof. Michael Heinrich

London 06.09.04

Dear partners,

Welcome back to the lab and office and I hope you all had a relaxing summer. Certainly, our Greek colleagues had a particularly exciting and enjoyable 17-day Olympic marathon.

As you all know the consortium only has another four months to go. Just to remind everyone, the crucial points for the next months include:

- Completion of the TIPs with the first draft due in two weeks
- All manuscripts as outlined in the minutes of the Milano meeting
- Preparation of the final report

The minutes of the last meeting in Milano were sent out in early August. Please note that we have not included them in this newsletter but sent it out as separate 'News'. This newsletter reports on the state of work mainly in WP 3,4,5. The next one can be expected by the end of October including updates to work packages 2,6 and 7.
Unfortunately there are no news on the financial evaluation of the second year, yet. As no further details were requested concerning the cost statements since June, we hope getting the next payment rate soon.

With my best regards
Michael Heinrich

Table of Content:

Work progress

Actual state of work

WP	Work Package title	Responsible participant	Partners involved	Person/ month	Start month	End Month	Deliverables No	State of work
	General administration of project	**1**	1	20	1	36	0.1 0.2	ongoing
1	**Identification, collection of food plants** Task1 Selection of local communities Task2 Identification of food plants Task3 Etnobiological study Task4 Database Task5 Development of a book and edition in local languages	**1**	 1,2,3 1.2.3 1.2.3 1 1.2 ,3	86	1	24	1.1 1.2 1.3 1.4	 completed completed completed completed (currently under review) ongoing
2	**Detailed socio-nutritional study** Task1 Development of data collection tools and guidelines Task2 Identification of cultural and social aspects Task3 Identification of anthropological aspects of the local nutrition patterns Task4 Clinical intervention study Task5 Social health and socio economical outputs	**3**	 1,2,3 1.2.3 1.2.3 3 1.2.3	110	1	36	2.1 2.2 2.3	 completed completed completed ongoing ongoing
3	**Primary screening of plant extracts** Task1 Primary assays Task2 Fractionation of the extracts Task3 Anti-oxidant activity Task4 Identification and structure elucidation of active principles Task5 Genotoxic test	**6**	 6 6 1 6 7	80	1	24	3.1 3.3 3.4	 completed completed completed completed completed
4	**Ageing-related disorders of the CNS** Task1 *In vivo* and *in vitro* experiments Task2 Patent protection of new molecules that may result from the research Task3 Publication of pharmacological results	**4**	4 (1.6)	66	3	36	4.1 4.2 4.3	 ongoing ongoing
5	**Cardiovascular ageing-related disorders** Task1 *In vivo* and *in vitro* experiments Task2 Patent protection of new molecules that may result from the research Task3 Publication of pharmacological results	**5**	5 (1,6)	66	3	36	5.1 5.2 5.3	 ongoing ongoing
6	**Anti-inflammatory and angiogenic activities of plant extracts** Task 1 Immunomodulatory and anti-inflammatory properties of the plant extracts Task 2 Angiogenic activities of plant extracts	**7**	1,3	36	13	30	6.1 6.2	 completed completed
7	**Molecular mechanisms of anti-inflammatory and anti-angiogenic effects of the tested plant extracts** Task 1 Effects on activation of transcription factors and stress-regulated kinases Task 2 Mechanisms of angiogenic activity detected in plant extracts	**7**	1,3	24	13	36	7.1	 ongoing ongoing

Completed
Ongoing in time
Ongoing with certain delay
Not yet started

Milestones completed

N.	Title	Description	Partic.	Rel. WPs	Expect. Month
MS 1	Availability of prioritised local food plants for further development into nutraceuticals (database)	• Database which will allow the processing of plants through WP3-WP5 and in the dissemination, suitable for the storage of information about: • 50-150 food botanical taxa (including less studied minor crop species and selected endemic non-cultivated taxa), with a good health potentiality, will be extracted in WP2 • Qualitative data about each plant: vernacular name, part used, processing, (possibly detoxification procedures), mode of preparation and quantitative data about the precise use of each taxa as food: frequency of use, taste properties	1.2.3	WP1	12
MS 2	Set of data on the social frameworks related to the nutritional behaviours in the areas selected	• Assessment of the psychological and anthropological factors important to consumers in the choice of specific dietary components • Comprehensive data on the social frameworks related to the nutritional behaviours in the areas selected • Health educational inputs for sustaining traditional dietary plant supplements intake which will sustain their marketing in the selected areas.	1.2.3	WP2	24
MS 2.1		• Selection of candidate species for a clinical intervention study focusing on the acute/ postprandial effect	all	WP1-3	24
NM 2.2		Decision about further development of lead species based on measurement of blood parameters (TC, TG, HDL, LDL, Apo A I, insulin, glucose, platelets aggregation, plasma resistance to oxidative stress [e.g. total antioxidant capacity (TAC), ox-LDL (lag-time), GSH, SOD], Homocysteine, Fibrinogen)	1.2.3	WP2	24
MS 3	Identification of extracts of food plant, fractions thereof and isolated compounds with activity in the primary tests	• Extracts of food plant, fractions thereof and isolated compounds with biological activities on one of the targets relevant to memory formation and metabolic diseases. • Exclusion of species with potential genotoxic effects	1.2.3	WP3	33
MS 5	Identification of nongenotoxic extracts exhibiting potent anti-inflammatory and/or anti-angiogenic effects	Formulation of nutritional compounds that may be useful in the inflammatory diseases	7	WP6 WP7	30

Next milestones

N.	Title	Description	Partic.	Rel. WPs	Expect. Month
MS 4	Identification of extracts, fractions thereof and isolated compounds (samples) improving brain functions with anti-oxidative properties.	• Identification of extracts, fractions thereof and isolated compounds (samples) improving brain functions with anti-oxidative properties, capability to reduce free radical generation, interaction with neuronal membranes, activity of anti-oxidative enzymes and improvement of signal transduction processes in peripheral systems and the CNS. • Formulation of extracts of food plant, fractions thereof and isolated compounds that can be further developed into nutritional supplements, for use especially in elderly. • Establishing if new nutraceuticals developed within the European Community have potential in vivo activities and suitable for commercial exploitation		WP4 WP5	36
MS 6	Identification of the molecular mechanisms of anti-inflammatory and anti-angiogenic activities of selected extracts	• Determination of the effect of extracts on the activity of selected transcription factors (NF-kB, AP1, STAT3, HIF-1, SP-1) and stress-regulated kinases (p38, JNK) • Determination of the effect of extracts on the synthesis of vascular endothelial growth factor	7	WP7 WP8	36

Workpackage 1

Revision database (Task 4)

After the meeting in Murcia the partner 1-3 agreed the revision of the database. The combined databases will serve for an analysis including all three regions and a joint publication on it. For a useful analysis the data will be summarized to a simplified scheme and transformed from access to excel. Responsible is Marco Leonti from the London group.

London group booklets (Task 5)

Due to Sabine's absence the work for the Galliciano Booklet for Southern Italy has been delayed. It can't be guaranteed that the book will be finished by Dec 2004.

The book on "Wild food plants of Castelmezzano and their traditional culinary uses "Piante alimentari locali e tradizioni popolari a Castelmezzano" (300 copies) is completed by Andrea Pieroni and planned for publication later this year by the Italian print office Coop BFS Pisa.

Murcia group booklet (Task 5)

The Spanish booklet will be completed by a chapter 6 with actual pharmacological information according the project outcome As decided during the Meeting in Milan all pharmacological groups sent short monographs of their hit plants (only Basel results are missing yet) to the London group. Marco and Michael actually collaborate with Diego for a useful edition of the pharmacological results.

Workpackage 3

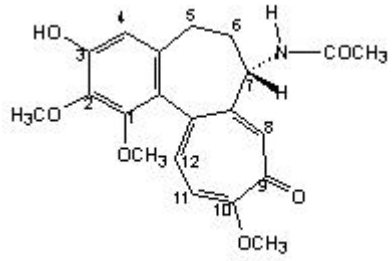
Database (D 3.3)

The database is not accessible on the DSM website anymore, as informed also during the Milan meeting. The excel file (CD) with all primary screening data will be distributed by Antoine de Saizieu, Basel. If any group didn't update their data yet, please do so sending him your last version.

Identification of isolated compounds (MS 3)

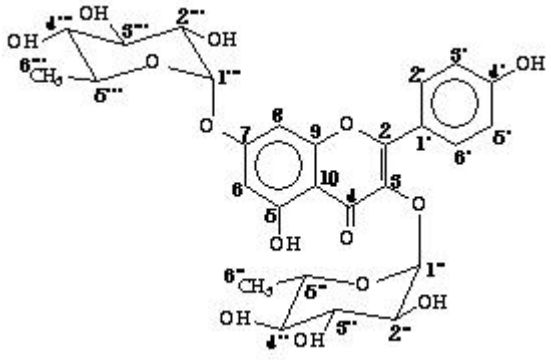
Marco Leonti, London

Phytochemical investigation of *Merendera montana* (*Liliaceae*). The fact that the colchicine-type alkaloid containing "macucas" is considered a food plant in Castilla-La Mancha and the fact that only few information on its phytochemistry were available encouraged us to start a phytochemical investigation.
In the framework of a diploma thesis apart from colchicine we found, 3-demethyl-colchicine, 3-demethyllumicolchicine, and the flavones apigenin and luteolin, which have been isolated previously.



3-demethylcolchicine, MW: 385.41

During the course of another diploma thesis we investigated the phytochemistry of *Reseda alba* (*Resedaceae*), which is used as a food plant by a Greek minority in Southern Italy. Only one compound, a glucosinolate, had previously been isolated from *R. alba*. In this work, five flavonoids have been isolated, one out of which has been identified as kaempferol-3,7-O-α-L-dirhamnoside and another as kaempferol 3-glucoside-7-rhamnoside.



kaempferol-3,7-O-α-L-dirhamnoside

Workpackage 4

Sebastian Schaffner, Frankfurt

Feeding study I and II summary

Regarding the in vivo testing of the extracts that were most active in different in vitro test systems we started the second feeding trial. Feeding study II was designed analogously to the previous study (described in newsletter 4). Briefly, young (3 month) and aged (18 month) female NMRI mice were feed with standard diet containing the extracts 1020, 1014 and 2025. One group received standard diet without extract for control. Daily intake of extract was estimated to 100 mg/kg b.w. For the test period of 3 months, mice had free access to food and water. At the end of the study tissue was collected. Antioxidative status measurement in blood samples, lipid peroxidation and antioxidative enzyme detection in brain was determined in the former study and will be repeated herein. Further, levels of radical oxidative species (ROS) will be will be determined in dissociated brain cells. As a new approach we will test the effects of antioxidative plant extract intake on DNA and protein damage (see underneath).

Feeding study I was finished in April. By now Total Antioxidative Capacity (TEAC) and hydroperoxide correlated oxidative stress (HPO) have been measured in blood samples of the rodents. Further, lipid peroxidation (MDA) levels and superoxide dismutase (SOD), glutathion peroxidase (GPx) and glutathion reductase (GR) activities have already been quantified. Preliminary data are presented below:

Preliminary results FSI

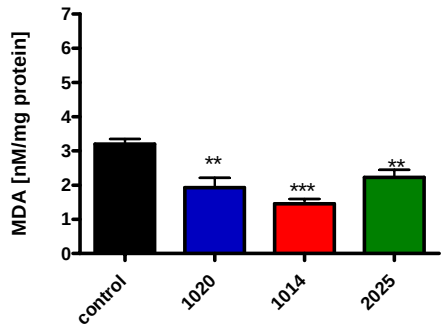
Extract administered effects were detected in different test systems.

Extract no.	TEAC	HPO	MDA	GPX	GR	SOD
1014	+	+	+	-	+	-
1020	+	+	-	+	-	-
2025	+	-	-	+	-	-

+ :probable effect (either positive or negative), - : no differences

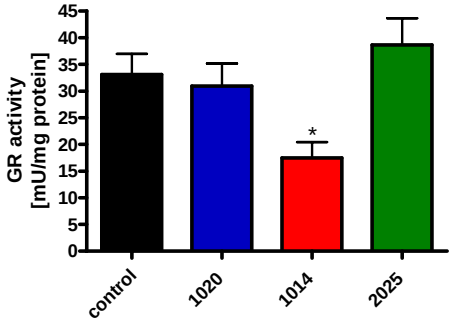
Activities of glutathion transferase and superoxid dismutase were uneffected.

Lipid peroxidation (brain)



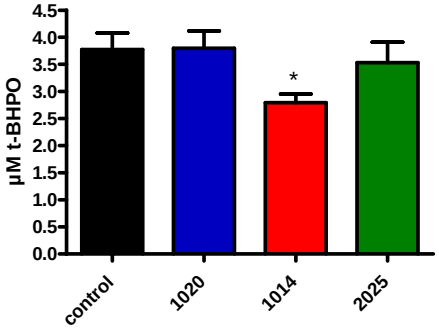
Effect of extracts on basal lipid peroxidation measured in mouse brain homogenates as concentration of MDA (nM/mg protein). Values are means + SEM of 4-8 NMRI mice. Basal level of MDA decreased with extract intake. ANOVA indicated a significant effect of extracts 1020 and 2025 (** $p < 0,01$) vs. control and of extract 1014 (*** $p< 0,001$) vs. control in young mice.

Glutathion reductase (brain)



Effect of extracts on the antioxidant enzyme activity of glutathione reductase (GR) in mouse brain. Enzyme activity was measured in brain homogenates from female NMRI mice. Values are means + SEM of 4-8 animals. Student`s t-test showed a significant reduction (* $p < 0,05$) of GR activity vs. control for extract 1014.

Hydroperoxid levels (blood)



Effect of extracts on the hydroperoxid levels in blood samples collected from treated mice. Hydroperoxid levels are expressed as tert-butylhydroperoxid equivalents. Values are means + SEM of 4-8 animals. Student`s t-test showed a significant reduction (* $p < 0,05$) of hydroperoxides vs. control for extract 1014.

New biochemical parameters to investigate

Originally, fluidity measurements in brain membranes were included in the research program. It is well known that aging and lipid peroxidation effect DPH-ansiotropy values. However, our in vitro studies ruled out that this parameter is not adequate to evaluate the effects of mediterranean food plants on membrane properties. Since we measured MDA levels that represent a valuable marker for oxidative membrane damage, we skip the DPH-anisotropy test. Instead of this we introduced the 8-OhdG - assay (ELISA) as an new test to estimate oxidative damage of DNA. Furthermore, we introduced the Oxiblot – Assay (OxiB) as an new test to estimate the effects of oxidative stress on structural protein changes.

New data on cellular mechanisms of oxidative stress led us to change our work plan: We replaced the measurement of intracellular calcium homeostasis against the measurement of changes in mitochondrial membrane potential (MP) and nitrite oxide-levels (NO). The membrane potential is detected with Rhodamine 123 (R123) which diffuses easily through the cell membrane and accumulates at the negative charged mitochondria membrane. NO-levels are detected with diaminofluorescein diacetat (DAF-2 DA). This dye is membrane permeable, the diacetat moiety is hydrolysed by esterases occurring within the cells. The resulted diaminofluorescein rapidly reacts with NO. The resulting fluorescent molecule indicates the levels of NO.

	OxyB	8-OHdG	MP	NO
tissue	brain, (liver)	brain	brain	brain
Study I	√	√		
Study II	√	√	√	√

New biochemical parameters

Feeding study II

Feeding period: 90 days
extract concentration: 100 mg extract/kg BW ▪ d
parameters: TEAC, HPO, ROS, membrane potential, NO-levels
(repetition of: lipidperoxidation and enzymes)

	18 month (aged)	3 month (young)
Extract 1020	10 females	8 females
Extract 1014	10 females	8 females
Extract 2025	10 females	8 females
control	10 females	10 females

Number of animals in study II

Biochemical parameters to investigate

	TEAC	HPO	MDA	GPX	GR	SOD	ROS
tissue	blood	blood	brain, liver (?)	brain, liver (?)	brain, liver (?)	brain, liver (?)	brain
Study I	√	√	√	√	√	√	
Study II	√	√	√	√	√	√	√

Biochemical parameters investigated

Outcome feeding study II

	18 month (aged)	3 month (young)
Extract 1020	4 females	8 females
Extract 1014	8 females	8 females
Extract 2025	8 females	8 females
control	5 females	10 females

Numbers of animals that complete study II

Study finished August, 10, 2004. Data analysis is still under processing.

Workpackage 5

Compounds in wild plants from the Mediterranean area favorably effect endothelial function, thus providing the basis for a cardioprotective action.

F. Visioli, S. Grande and C. Galli, Milan

Among the many ethanolic extracts prepared from plant specimens collected in the context of the EU Grant Local Food Nutraceutical, those obtained from Southern Italy, as well as those from Greece had the highest content in polyphenols. These compounds, in addition to being involved in the regulation of plant physiology, in response to the environmental conditions, when ingested with the diet are able to

favorably affect various processes in biological systems. Some of these activities are generally termed as "antioxidant", referring to the inhibition of chemical reactions activated by the production of reactive oxygen species (ROS), which are cytotoxic. In reality, several processes, sequentially or simultaneously activated by ROS (e.g. DNA damage, unwanted enzyme activation and generation of cellular mediators, etc.) are controlled by plant derived compounds.

The spectra of components in the extracts from *C. cardunculus* and *T. pulegioides* from Southern Italy, particularly rich in polyphenols, were assesses by the use of HPLC-coupled with ESI and DOD detectors, and it was found that luteoline glycoside, apigenin, rosmaric, asiatic and ursolic acids were the major compounds.

The first impact of any ingested compound when entering the body is on the lining of the vascular system, e.g. the endothelium. Experimental research over the last decade has allowed developing diversified methodogies aimed at assessing "biochemical" markers of endothelial metabolism in cultured cells as well as at evaluating "physiological" parameters relevant to the function of the whole vessel wall in integrated systems (e.g. isolated animal vessels). At the same time a number of studies have indicated that endothelial function is affected, especially in the postprandial phase, by what we ingest with the diet, and that "endothelial dysfunction" can be induced by the intake of certain dietary components, leading to the subsequent onset of CV diseases, over time. In contrast, the ingestion of "antioxidants" and other bioactive compounds may have protective effects.

Our research interests were therefore focused on the evaluation of the activities of the extracts from the two above indicated plants, on the production of "protective" endothelial mediators (namely nitric oxide, NO and prostacyclin, PGI2) in cultured porcine aortic endothelial cells (PAEC). NO is produced through the activation of the endothelial enzyme eNOS is a potent vasorelaxant, while PGI2, generated through the sequential activations of a phospholipase A2, a Cyclooxygenase (COX) and a prostacyclin synthase is a vasorelaxant and also a potent inhibitor of platelet aggregation.

We have also evaluated the effects on the function of aortic vessel walls obtained from experimental animals, assessed as the ability of inducing the relaxation of a contracted vessel (rat aorta). In order to do so, in addition to the use of the ethanolic extracts generally prepared from plants for testing the effects on "biochemical" parameters in cultured cells, we have prepared aqueous extracts (decoctions) of the same plants. These preparations vs. the ethanolic ones did not contain the lipid soluble, e.g. fatty acids, compounds that we found to interfere with the bioactive components in the assessment of "functional" effects.

The combined measurements on cultured endothelial cells and on vessel walls provide information on the integrated (biochemical and functional) mechanisms for the effects of the extracts under testing on the vascular system. The extracts, from the two plants when incubated with PAOC, very potently enhanced both the production of NO and of PGI2 as shown in Fig 1 and 2. In addition the aqueous extracts, very rich in the listed water-soluble flavonoids, induced (not shown) the relaxation of contracted rat aortas, indicating that favorable biochemical effects were associated with favorable changes in vessel function.

In conclusion, extracts from typical wild plants traditionally consumed in Southern Europe are able to "beneficially" affect some key processes in the protection of endothelial as well as of whole vessel function.

Publication intentions

Please find attached to this newsletter a form for publication intentions (as decided during the Milan meeting). Please return the filled form together with the TIP 2 and 3 forms (deadline 15 Sep), were the majority of the considered publications will be mentioned anyway. Later, the coordinator will distribute the summarized document.

Reminder

Deliverables

Please find below the deliverables of the Technical Annex (TA), which actually are the basis for the evaluation of the final report. (together with the milestones and work package descriptions, see work progress tables above) Four months before the project finishes, the responsible partner of every work package should justify the realistic accomplishment of the work according the TA. In case of foreseeable deviations please contact the coordinator. Please remember also to calculate the total costs before as depending on the volume (20 %) those deviations have not only to be reported to the commission but also agreed by Brussels before.

WP1 (responsible partner Murcia)

D 1.3 Book on the local use of food plants	Dec 04

WP2 (responsible partner Athens)

D 2.3 Reports & scientific publication of the epidemiological and nutritional pattern of the studied areas	Dec 04
N 2.2. Interim report on outcome of clinical intervention study focusing on the acute/ postprandial effect	**Jul 04**
N 2.3. Final report of the study and publication summarizing the findings of the clinical study	Dec 04

WP3 (responsible partner London)

D 3.1 Botanical validated extracts, fractions thereof and isolated compounds, which may also be used by other research groups (if they are of no interest to the consortium).	Feb 04
D 3.2 Samples (anticipated ca.150) for the compound library to be used for the HTS systems (partner 6).	Mar 04
D 3.3 Storage of all data in a database already established by partner 6.	**Mar 04**
D 3.4 *final report* with the results of WP 3. Subsequently, publication will be elaborated in those cases where patent protection is not the primary interest	**Jun 04**

WP4 (responsible partner Frankfurt)

D 4.2 Prioritised plant samples with known mechanism (CNS models) for further development	**Jun 04**
D 4.3 Application for patents (or report) on established local food species evaluated *in vitro*	Dec 04

WP5 (responsible partner Milano)

D 5.2 Prioritised extracts from the ethnobotanical collections with known mechanism for further development	**Jun 04**
D 5.3 Application for patents (or report) on extracts from the ethnobotanical collections with validated *in vitro* and *in vivo* activity	Dec 04

WP6 (responsible partner Krakow)

D 6.2 Validated methods for screening plant extracts in respect of anti-inflammatory and anti-angiogenic properties	**Jun 04**

WP7 (responsible partner Krakow)

D 7.1 Completion of assays for plant extracts-induced metabolic changes in activation of transcription factors and angiogenic activities, including report or patent protection on novel inducers of metabolic changes	Dec 04

Next newsletter

There will be another newsletter by the end of October, which will include an update on the database compilation (WP1 and 3), the TIP submission, all the manuscripts, details on the Krakow meeting, and actual progress reports with major emphasis on WP2, WP6 and WP7.

Next meeting

Final Meeting and Symposium of the Consortium in Krakow **Dec 2-3**

Next documents to be submitted

TIP part 2	**15 Sep**	London, Murcia, Frankfurt, Basel
TIP part 3	**15 Sep**	All partners
newsletter updates	**20 Oct**	Athens, Krakow, Basel
Krakow contributions	**20 Oct**	All partners
Final reports	**15 Nov**	All partners