EUROPEAN COMMISSION
Research Executive Agency
Marie Curie Actions – International Research Staff Exchange Scheme

Project No: 316067

Project Acronym: HERBAL PROTECTION

Project Full Name: Studies on some herbal additives giving partial protection against toxic or immunosuppressive effects of some mycotoxins and improving wound granulation

Marie Curie Actions

Periodic Report

Period covered: from 01/01/2015 **to** 31/12/2016

Period number: 2nd

Start date of project: 01/01/2013

Project coordinator name:
Prof. Stoycho Stoev

Version: 1

Date of preparation: 09/01/2017

Date of submission (SESAM): 18/01/2017

Duration: 48

Project coordinator organisation name:
TRAKIA UNIVERSITY

Periodic Report

1. PROJECT PERIODIC REPORT

Grant Agreement number:	316067
Project acronym:	HERBAL PROTECTION
Project title:	Studies on some herbal additives giving partial protection against toxic or immunosuppressive effects of some mycotoxins and improving wound granulation
Funding Scheme:	FP7-MC-IRSES
Period report:	2nd
Period covered - start date:	01/01/2015
Period covered - end date:	31/12/2016
Name, title and organisation of the person in charge of the project for the beneficiary(ies):	Prof. Stoycho Stoev TRAKIA UNIVERSITY
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Project website address:	http://www.uni-sz.bg/node/1601

2. DECLARATION BY THE PROJECT COORDINATOR

I, Prof. Stoycho Stoev, as person in charge of the project (316067, HERBAL PROTECTION), for the beneficiary(ies), hereby confirm that:

- The attached periodic report represents an accurate description of the work carried out in this project for this reporting period;

- The project:

( ) has fully achieved its objectives and technical goals for the period;

(o) has achieved most of its objectives and technical goals for the period with relatively minor deviations;

( ) has failed to achieve critical objectives and/or is not at all on schedule.

- The project Website is up to date (if applicable);

- To my best knowledge, the financial statements which are being submitted as part of this report are in line with the actual work carried out and are consistent with the report on the resources used for the project and if applicable with the certificate on financial statement;

- The beneficiary(ies), in particular non-profit public bodies, secondary and higher education establishments, and research organisations, have declared to have verified their legal status. Any changes have been reported under section 3 (Project Management) in accordance with Article II.2.f (Monobeneficiary) or with Article II.3.f (Multibeneficiary) of the Grant Agreement.

3. SUMMARY OF THE SECONDMENT OF RESEARCHERS DURING THE REPORTING PERIOD

Beneficiary: TRAKIA UNIVERSITY

WP n°	First name of the Researcher	Last name of the Researcher	Date of Birth	Gender	Type	Seconded To	Seconded To(Short Name)	Seconded To (Country)	Start date of secondment	End date of secondment	EU Contribution / fellow-month (€)	No. of full-time equivalent months covered by this secondment during this reporting period	Total EU Contribution (€)
5	Kiril Kostadinov	Dimitrov	08/10/1986	Male	ESR (<4 years)	UNIVERSITY OF JOHANNESBUR G	UJ	ZA-South Africa	10/11/2016	31/12/2016	2100	1.70	3570.00
7	Ivan Dinev	Ivanov	15/05/1961	Male	ER (4-10 years)	UNIVERSITY OF JOHANNESBUR G	UJ	ZA-South Africa	13/11/2016	27/12/2016	2100	1.47	3087.00
2	Veselin Asenov	Ivanov	11/05/1968	Male	ER (4-10 years)	RHODES UNIVERSITY	RU	ZA-South Africa	21/11/2016	30/12/2016	2100	1.30	2730.00
3	Ivan Dinev	Ivanov	15/05/1961	Male	ER (4-10 years)	UNIVERSITY OF JOHANNESBUR G	UJ	ZA-South Africa	01/05/2016	30/07/2016	2100	2.97	6237.00
3	Veselin Asenov	Ivanov	11/05/1968	Male	ER (4-10 years)	RHODES UNIVERSITY	RU	ZA-South Africa	01/01/2015	28/01/2015	2100	0.90	1890.00
7	Yanka Dimitrova	Karamalakova	26/07/1978	Female	ER (4-10 years)	MINISTRY OF DEFENCE	DRDO	IN-India	01/07/2015	24/09/2015	2100	2.80	5880.00
7	Galina Dimitrova	Nikolova	30/06/1975	Female	ER (4-10 years)	MINISTRY OF DEFENCE	DRDO	IN-India	01/07/2015	24/09/2015	2100	2.80	5880.00
4	Miroslav Georgiev	Stefanov	30/11/1960	Male	ER (4-10 years)	RHODES UNIVERSITY	RU	ZA-South Africa	30/09/2016	30/12/2016	2100	3.00	6300.00
2	Miroslav Georgiev	Stefanov	30/11/1960	Male	ER (4-10 years)	UNIVERSITY OF JOHANNESBUR	UJ	ZA-South Africa	17/06/2016	17/07/2016	2100	1.02	2142.00

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2	Miroslav Georgiev	Stefanov	30/11/1960	Male	ER (4-10 years)	MINISTRY OF DEFENCE	DRDO	IN-India	15/05/2016	16/06/2016	2100	1.08	2268.00
5	Georgi	Valentinov Terziev	18/04/1988	Male	ESR (<4 years)	UNIVERSITY OF JOHANNESBUR G	UJ	ZA-South Africa	30/09/2016	19/11/2016	2100	1.67	3507.00
Total no. of full-time equivalent months covered by this secondment during this reporting period:												20.71	
Total EU Contribution for this Beneficiary in euros (not including the contribution to third countries secondments):													43491.00

Beneficiary: KAPOSVARI EGYETEM

WP n°	First name of the Researcher	Last name of the Researcher	Date of Birth	Gender	Type	Seconded To	Seconded To(Short Name)	Seconded To (Country)	Start date of secondment	End date of secondment	EU Contribution / fellow-month (€)	No. of full-time equivalent months covered by this secondment during this reporting period	Total EU Contribution (€)
3	Nagy	Gabor	30/08/1972	Male	ER (4-10 years)	UNIVERSITY OF JOHANNESBUR G	UJ	ZA-South Africa	25/05/2016	23/08/2016	2100	2.97	6237.00
Total no. of full-time equivalent months covered by this secondment during this reporting period:												2.97	
Total EU Contribution for this Beneficiary in euros (not including the contribution to third countries secondments):													6237.00

Partner: MINISTRY OF DEFENCE

WP n°	First name of the Researcher	Last name of the Researcher	Date of Birth	Gender	Type	Seconded To	Seconded To(Short Name)	Seconded To (Country)	Start date of secondment	End date of secondment	EU Contribution / fellow-month (€)	No. of full-time equivalent months covered by this secondment during this reporting period	Total EU Contribution (€)
3	Manish	Adhikari	03/04/1985	Male	ER (4-10 years)	TRAKIA UNIVERSITY	TU	BG-Bulgari a	01/01/2015	19/02/2015	2100	1.68	3528.00
4	Prerna	Agarwal	30/06/1989	Female	ER (4-10 years)	TRAKIA UNIVERSITY	TU	BG-Bulgari a	01/01/2015	19/02/2015	2100	1.68	3528.00
Total no. of full-time equivalent months covered by this secondment during this reporting period:												3.36	
Total EU contribution for this partner. It will be distributed among the hosting beneficiaries:													7056.00

Partner: RHODES UNIVERSITY

WP n°	First name of the Researcher	Last name of the Researcher	Date of Birth	Gender	Type	Seconded To	Seconded To(Short Name)	Seconded To (Country)	Start date of secondment	End date of secondment	EU Contribution / fellow-month (€)	No. of full-time equivalent months covered by this secondment during this reporting period	Total EU Contribution (€)
3	Bertha	Chithambo	27/05/1966	Female	ER (4-10 years)	TRAKIA UNIVERSITY	TU	BG-Bulgari a	03/11/2016	02/12/2016	2100	1.00	2100.00
3	Hilary Ihesinaulo	Ezuruike	20/10/1968	Male	ESR (<4 years)	TRAKIA UNIVERSITY	TU	BG-Bulgari a	21/09/2015	15/12/2015	2100	2.82	5922.00
3	Derek Tantoh	Ndinteh	07/05/1975	Male	ER (4-10 years)	KAPOSVARI EGYETEM	UNIKAPOS	HU-Hungary	08/07/2016	07/10/2016	2100	3.00	6300.00
3	Xavier Siwe	Noundou	25/04/1975	Male	ER (4-10 years)	TRAKIA UNIVERSITY	TU	BG-Bulgari a	23/09/2016	22/11/2016	2100	2.00	4200.00
3	Xavier Siwe	Noundou	25/04/1975	Male	ER (4-10	KAPOSVARI EGYETEM	UNIKAPOS	HU-Hungary	19/05/2015	10/07/2015	2100	1.74	3654.00

					years)								
Total no. of full-time equivalent months covered by this secondment during this reporting period:												10.56	
Total EU contribution for this partner. It will be distributed among the hosting beneficiaries:													22176.00

Partner: UNIVERSITY OF JOHANNESBURG

WP n°	First name of the Researcher	Last name of the Researcher	Date of Birth	Gender	Type	Seconded To	Seconded To(Short Name)	Seconded To (Country)	Start date of secondment	End date of secondment	EU Contribution / fellow-month (€)	No. of full-time equivalent months covered by this secondment during this reporting period	Total EU Contribution (€)
6	Mbali Lomalangeni	Webb	23/09/1991	Female	ESR (<4 years)	KAPOSVARI EGYETEM	UNIKAPOS	HU-Hungary	08/07/2016	06/10/2016	2100	2.97	6237.00
Total no. of full-time equivalent months covered by this secondment during this reporting period:												2.97	
Total EU contribution for this partner. It will be distributed among the hosting beneficiaries:													6237.00

Totals per beneficiary (to be encoded in FORMs C):		
Beneficiary(short name)	Total EU Contribution	Total no. of full-time equivalent months covered by this secondment during this reporting period
TRAKIA UNIVERSITY	62769	29.89
KAPOSVARI EGYETEM	22428	10.68
Totals:	85197.00	40.57

Totals per partner (for information only):		
Partner(short name)	Total EU Contribution	Total no. of full-time equivalent months covered by this secondment during this reporting period
MINISTRY OF DEFENCE	7056.00	3.36
RHODES UNIVERSITY	22176.00	10.56
UNIVERSITY OF JOHANNESBURG	6237.00	2.97
Totals:	35469.00	16.89

4. PUBLISHABLE SUMMARY

Publishable summary:

This research has pointed towards the use of natural compounds in herbs as universal protectors or mitigators against toxicity of some deleterious agents such as mycotoxins and/or radiation. Such protective effects were seen for naturally occurring dietary ingredients, mainly containing flavonoids. Within the time-frame of the second 2 years some herbs from South Africa and India were studied for their biological activity and protective effects using various cell lines or animal modells. Some of the studied herbs such as Centella asiatica, Withania somnifera, Silybum marianum, Tinospora cordifolia, Glycyrrhiza glabra, Stem bark of the trees Piptadenastrium africanum, Haberlea rhodopensis, Curcuma Longa (Turmeric) and Ginger (the rhizome of the Zingiber officinale) were found to have some target anti-bacterial, anti-fungal, anti-inflammatory, immuno-stimulating and antioxidative activities as well as protective effects on kidneys and liver or to be useful for wound healing. Some of them were found to have a high content of flavonoids and to ameliorate gamma-radiation-induced lesions or to protect against deleterious effects of some mycotoxins. 16 herbal extracts were analyzed for the presence of bio-active constituents and antioxidant potential. The bioactivity analysis of selected herbs revealed following fingerprints: Anti-lipid peroxidation (60-70%); Nitric Oxide Scavenging (50-70%); Site Specific Hydroxyl Radical Scavenging (70-80%); Non-Site Specific Hydroxyl Radical Scavenging (30-40%). Qualitative analysis of classes of phyto-chemicals revealed following ranges: Alkaloids (Moderate to Extremely High); Tannins (Very Low to Low); Terpenoids (Moderate to Extremely High); Saponins (Low to Moderate); Glycosides (Moderate to High); Anthraquinones (Very Low to Moderate); Proteins (Moderate or otherwise absent). The phytochemical fingerprint analysis of these herbs revealed: Phenolic content of herbs ranges from 60 to 85% with respect to gallic acid used as standard equivalence; Flavonoid content of herbs ranges from 30 to 70% with quercetin used as standard equivalence; On the basis of the above results 7 herbs were further screened in order to test their efficacy against targeted mycotoxins: Glycyrrhiza glabra, Tinospora cordifolia, Zingiber officinale, Curcuma longa, Centella asiatica, Silybum marianum, Withania somnifera.

Various in vivo or ex vivo enzymatic and non-enzymatic experiments e.g., Catalase, Glutathione Reductase, Glutathione Peroxidase and Superoxide dismutase, etc at liver and intestine tissues revealed the protective effects of some target herbs against mycotoxin-induced toxicity.

The algorithm for assessing radio protective potential of plant extracts and natural products was assessed via some target in vitro and in vivo tests and clinical trials. The mechanisms of radio protective action of the tested extracts and natural products was analysed.

A number of formulation approaches have been employed to increase the solubility and oral

absorption of some herbal extracts and products and subsequently to enhance their bioavailability and therapeutic activity.

The antioxidant capability of the resulting plant extracts against a stable radical DPPH was evaluated and the most of plant extracts showed high efficiency in the DPPH test.

The following Indian herbs Tinospora cordifolia (in dose 300 mg/kg bw or 4000 ppm via the feed) and Glycyrrhiza glabra (in dose 400-600 mg/kg bw or 6600 ppm via the feed) and following South African herbs: Centella asiatica (in dose 300-400 mg/kg bw or 4600 ppm via the feed), Withania somnifera (in dose 200-400 mg/kg bw or 4000 ppm via the feed) and Silybum marianum (in dose 80 mg/kg bw or 1100 ppm via the feed) appeared to have a good protective effect in broiler chick (breed ROSS) against various toxic effects of mycotoxin ochratoxin A on the body weight, relative organ weight, biochemical indices and humoral immune response. A hepatoprotective effect was seen for Tinospora cordifolia and Glycyrrhiza glabra being stronger for chicks additionally supplemented with Glycyrrhiza glabra as can be seen from the pathomorphological findings and the lower levels of ASAT and ALAT. Protective effects of herbal feed additives Silybum marianum or Withania somnifera and slightly of Centella asiatica against the growth inhibitory effect of ochratoxin A and associated immunosuppression and biochemical or pathomorphological changes were seen, e.g. protective effects on the kidneys (strongest for Silybum marianum) and/or liver. The strong immunesuppressive effect of OTA on humoral immune response against Newcastle disease was completely prevented in chicks given the herbal additives Withania somnifera or Silybum marianum, which was additionally supported by the higher relative weight of immunocompetent (lymphoid) organs in the same chicks. The same herbs or appropriate mixture between them could be used as a practical approach for safely utilizing of OTA-contaminated feed.

Nanoformulation of Silymarin with higher efficacy has been developed. SNEDDS (Self Nanoemulsifying Drug Delivery Systems) was prepared to increase the solubility and oral absorption for achieving better bioavailability and therapeutic activity of Silymarin. The radioprotective efficacy and preliminary studies against mycotoxin toxicity revealed that Silymarin nanoemulsion has promising results better than the parent silymarin compound. The radioprotective efficacy of Silymarin as a dietary supplement comprises of a mixture of flavonolignans containing silybin (main constituent), isosilybin, silychristin, silydianin and taxifoline commonly found in the dried fruit of milk thistle plant Silybum marianum. It can reduce gamma-radiation-induced micro nuclei formation and reactive oxygen species levels, apotosis and DNA damage (as measured by Comet assay and flow-cytometry) and mitochondrial membrane disruption. The silymarin nanoemulsion-pretreated (10µg/ml) irradiated group (Balb/c mice) showed lower frequency of apoptotic bodies of human embryonic kidney (HEK) cells as compared to radiation alone group. Survival studies using Balb/c mice confirmed that silymarin exhibits maximum protection at 50 mg/kg b/w against 9 Gy gamma-irradiation. Pre-irradiated treatment with silymarin could restore total lymphocyte counts (TLC) by the 15th day to normal. Based on the series in vivo and in vitro (MTT assay and Annexin V-PI studies, Comet assay and Flow-cytometry) studies, the analysis of data revealed that there is a shift in antioxidant balance upon administration of silymarin that leads to radioprotection. Protection against radiation-induced cell-death and DNA damage by silymarin could be attributed to a reduction in ROS induced by gamma-radiation. In vitro and in vivo experiments showed that silymarin is a promising, effective and safe radiation countermeasure agent and has potential for use during nuclear/radiological emergencies. Our results have clearly shown that the radioprotective efficacy of silymarin nanoformulation is better than silymarin parent compound and preliminary studies indicate its potential ability to reduce mycotoxin-induced toxicity. Therefore, nanosilymarin could be considered as useful source for mitigating both radiation and mycotoxin-induced toxicity warranting further studies to validate its efficacy in in vivo models.

EPR in vitro spectroscopy studies demonstrated that the naturally isolated Piptadenastrium africanum and Haberlea rhodopensis extracts exhibited well expressed DPPH scavenging capacity either before or after UV irradiation. In conclusion, we suggest that further detailed EPR in vitro and

in vivo studies for possible application of those extracts as potential radical scavengers and UV protectors in experimental animal models have to be carried out.

The binding ability of ochratoxin A using nano-enabled materials to mitigate exposure was also evaluated. All tested sample materials exhibited strong binding affinity toward OTA in solution. The use of these nanoparticles as feed additives in ameliorating the toxicity of OTA in animals and humans seemed promising. Further studies using some animal models are still required to ascertain the potentials of these materials for use as OTA binders.

Chitosan nanoparticles functionalized with plant extracts for the inhibition of the toxic effects of aflatoxin B1 and ochratoxin A were evaluated (green nanotechnology) with possible applications in preventing damages caused by these mycotoxins with the aim to improve food safety and boost human and animal health. The chitosan nanoparticles with extracts from medicinal plants (Menta Longifolia and Leonotis leonurus) were synthesised and characterised. The antioxidant ability of extracts was evaluated before being incorporated into chitosan using DPPH radical scavenging assay.

Protective effects of samples from leaves and stem bark of Erythrina caffra were found via MTT assay (cell viability method) on the lymphocyte cells in the presence of T-2 toxin.

Millettia macrophylla was found to have estrogenic effects and to prevent postmenopausal osteoporosis in Wistar rats. The identification of its secondary metabolites (13 metabolites) and the evaluation of their estrogenicity and cytotoxicity toward tumoural cells was also done.

The extracts or whole powder from South African herbs Centella asiatica, Withania somnifera, Silybum marianum and Indian herbs Glycyrrhiza glabra, Tinospora cordifolia, Ginger (the rhizome of the Zingiber officinale) and Curcuma Longa (Turmeric) were found to have wound-healing activity and/or anti-inflammatory activity and/or antibacterial or antifungal activities in the form unguents or sprays.

The antibacterial activity of the medium polar extracts of T. potatoria leaves and stem bark was found against Mycobacterium smegmatis. The compounds possibly contributing to this activity, and which may therefore be promising precursors to be used for the development of novel anti-TB drugs were established. Seven compounds were isolated from the medium polar extract [MeOH/DCM (1:1, v/v)] of T. potatoria stem bark. Two novel secondary metabolites named tetraceranoate and N-hydroxy imidate-tetracerane were isolated and identified. Tetraceranoate exhibited the best activity against M. smegmatis with a minimum inhibitory concentration (MIC) of 7.8 μg/mL, while β-stigmasterol, betulinic acid and betulin showed appreciable anti-mycobacterial activity (MIC 15 μg/mL). The isolated compound tetraceranoate showed antibacterial activity against M. smegmatis as high as rifampicin (one of a three drug regimen recommended in the initial phase short-course anti-tuberculosis therapy). Thus, tetraceranoate might be an interesting target for systematic testing of anti-TB treatment and management. This finding supports the use of T. potatoria in African traditional medicine for the treatment of tuberculosis related symptoms.

The leaves and stems of A. cordifolia exhibited varied antibacterial activity against four Gram-positive bacteria, i.e. Bacillus cereus ATCC11778, Enterococcus faecalis ATCC 29212, Staphylococcus aureus ATCC 25923 and S. saprophyticus ATCC 15305, as well as four Gram-negative bacterial strains, i.e. Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Moraxella catarrhalis ATCC 23246 and Proteus mirabilis ATCC 43071. Seven constituents [stigmasterol (1), stigmasta-4,22-dien-3-one (2), friedelin (3), friedelane-3-one-28-al (4), 3-O-acetyl-aleuritolicacid (5), 3-O-acetyl-erythrodiol (6) andmethyl-3,4,5-trihydroxybenzoate(methylgallate) (7)] were isolated from the stem MeOH extract of A. cordifolia. All these compounds displayed some antibacterial activity against the eight pathogens with highest activity against S. saprophyticus (2 mg/ml). The study demonstrated that the antibacterial activities of A. cordifolia extracts may be due to the presence of the seven isolated compounds, where compounds 3–6 showed the best activity. The observed activity against gastrointestinal, skin, respiratory and urinary tract pathogens supports the traditional use for the treatment of such ailments.

The investigation of the protective effect of the extracts derived from the plants Gunnera perpensa

and Hydnora abyssinica against the mycotoxin T-2 revealed no significant protection.

Studies on in-vitro efficacy of herbal extracts against Ochratoxin A using Normal Kidney Epithelial cells (NKE) and tumorigenic Kidney cell line (ACHN) revealed antioxidant potential of the most target extracts. NF-κB activation ability of 6 herbal extracts with radiation was determined on Lac-Z reporter cells and two extracts were found to have highest activity.

Studies on ex-vivo or in vivo efficacy of herbal extracts [RDP03, RDP06, RDP010, RDP09 and RDP011, e.g. Zingiber officinale, Tinospora cordifolia, Curcuma longa, Glycyrrhiza glabra extract] against Ochratoxin A with the help of EPR revealed a high efficiency. It was found that the levels of oxidative stress markers reduced significantly on addition of extract RDP03. In conclusion, we consider further more detailed in vitro and in vivo studies for possible application of those extracts as potential radical scavengers and protectors against different environmental stress causing oxidative damage.

Studies on influence of ochratoxin-A and an extract of Tinospora cordifolia against biochemical and oxidative changes in mice spleen tissue homogenates using EPR spectroscopy revealed that combination of OTA with oral administration of Tinospora cordifolia extract led to significant improvement in the levels of oxidative stress biomarkers in mice spleen. It seems that Tinospora cordifolia extract behaves as a good scavenger of ROS and RNS and might find application in the pharmaceutical and food industry as a protector against various diseases, e.g. mycotoxicoses.

Studies on protective effect of two essential oils isolated from Rosa damascene Mill. and Lavandula angustifolia Mill, and two classic antioxidants against L-dopa oxidative toxicity induced in healthy mice revealed that combining the L-dopa therapy (in the Parkinson's disease treatment) with antioxidants can reduce related side effects and provide symptomatic relief. The natural antioxidants can be isolated from any plant parts such as seeds, leaves, roots, bark, etc., and their extracts riched in phenols can retard the oxidative degradation of the lipids, proteins and DNA. Thus, study suggests that combination of essential oils (Rose oil and Lavender oil), Vitamin C and Trolox with L-dopa can reduce oxidative toxicity, and may play a key role in ROS/RNS disarm.

Studies on ex vivo effect of Glycyrrhiza glabra root extract on some "real time" biomarkers of oxidative stress via EPR spectroscopy revealed that Glycyrrhiza glabra (Licoric) exhibited good anti-inflammatory, antiviral, antimicrobial, antioxidative, anticancer, immunomodulatory, hepatoprotective and cardio- protective properties and excellent ex vivo radical scavenging capacity, which are relevant to radioprotection. It was established that in almost all organs of the treated mice the levels of biomarkers tested were close to those of the untreated controls. Significantly lower levels of nitrite and ascorbate radicals were measured only in the spleens and the hearts of the treated mice compared to controls. This EPR ex vivo study characterizes Glycyrrhiza glabra water extract as a good antioxidant.

The anticancer potential of the dichloromethane / methanol extract of Crateva adansonii stem barks was investigated using human breast cancer cell and 7,12 dimethylbenz(a) anthracene (DMBA)-induced mammary tumorigenesis model in rats The results suggest that the C. adansonii extract may possess antitumor constituents, which could combat breast cancer and prevent chemically-induced breast cancer in rats. C. adansonii extract significantly ($p<0.001$) revealed in vivo the reduction of the cumulative tumour yield (87.23%), total tumour burden (88.64%), average tumour weight (71.11%) and tumour volume (78.07%) at the dose of 75 mg/kg as compared to DMBA control group. This extract showed a moderate hyperplasia at the dose of 75 mg/kg while at 300 mg/kg no significant change was noted as compared to DMBA group. It protected rats from the DNA alteration induced by DMBA and increased antioxidant enzymes activities in mammary gland tissue homogenates. In addition, Ultra-High Performance Liquid Chromatography / ESI-QTOF-Mass Spectrometry analysis of C. adansonii extract detected structure-related of many well-known anticancer agents such as flavane gallate, flavonol, phenylpropanoïds, sesquiterpene derivatives, gallotannins and lignans. The LD50 of C. adansonii was estimated to be greater than 5000 mg/kg.

The transfer of knowledge and training activities (workshops) were done in various work

packages via the following activities:

- Training courses or specializations in different areas of research organized by various participants in different countries for receiving target skills.
- Screening of Herbal extracts for their anti-toxin efficacy performed on both normal and transformed cells.
- Standardization of bioassay protocols to evaluate nutraceutical standardization; antioxidant activity in both lipid and aqueous phase, free radical induced flux and; ex vivo systems for anti-lipid per-oxidation potential. These assays are used to standardize the nutraceuticals for its efficacy, which reduces with time (due to varied storage conditions). Such assays were carried out jointly and necessary training done.
- In silico biprospection model: A standardized mathematical model developed in house at the laboratory has been shared and necessary training imparted to use this model for selection of nutraceuticals based of multi-parametric based matrix analysis.
- Process standardized for herbal preparation preventing loss of thermolabiles compounds was shared and jointly performed for development of multiple solvent-system based nutraceuticals.
- The extraction of plant materials and compound isolation in Rhodes University (South Africa) was carried out with participation of visiting Marie Curie fellows by using various chromatographic techniques including low pressure column chromatography, preparative thin layer chromatography, high pressure liquid chromatography, high speed counter current chromatograhopy.
- Characterization of plant metabolites in Rhodes University (South Africa) was carried out with participation of visiting Marie Curie fellows by using nuclear magnetic resonance (NMR), Fourier Transform Infrared Spectroscopy (FTIR), ultraviolet (UV), elemental analyses (EA), Mass Spectroscopy (MS), Raman spectroscopy (RS), Mossbauer analyses (MA), etc.
- Training courses and acquired skills of visiting Marie Curie fellows in University of Johannesburg (South Africa) was realized in the extraction of active medicinal plant components and characterizing them using various chromatographic techniques including TLC and GC-MS/MS, MTT assay and Comet assay.
- The extraction and characterization of plant materials and compound isolation in TU (Bulgaria) was carried out with participation of visiting Marie Curie fellows by using EPR (Electron Paramagnetic Resonance) and NIRS (Near Infrared Reflectance Spectroscopy) on “Fiber Optic Spectrometer”, etc.
- Participation in various in vitro or in vivo experiments and exchange of knowledge or receiving some experience in various technics such as magnetic resonance imaging and spectroscopy, positron emission tomography, MTT assay, EPR (Electron Paramagnetic Resonance), NIRS (Near Infrared Reflectance Spectroscopy), DPPH radical scavenging assay, ABTS diamonium salt radical cation decolorization test is also used as a radical scavenging test, Comet assay, Annexin V-PI (propidium iodide) studies, flow-cytometry, etc.

5. GENERAL PROGRESS OF THE PROJECT

Description of the modifications:

All of the objectives and the tasks are achieved. There are only some amendments in regards to the time and destination of some secondments between the partners and beneficiaries, because of some organization changes and a subsequent transfer of some secondments and tasks from the partner DRDO to the beneficiary TU as follow:

-Work package 1 – without changes and all the tasks and scientific objectives were achieved

-Work package 2 – without changes and all the tasks and scientific objectives were achieved. A

secondment from TU was postponed in order to collect some more Himalayan herbs appeared to have a pronounced protective or healing effect on animals.

-Work package 3 – without changes and all the tasks and scientific objectives were achieved. Some secondment from each of the partners were postponed for the last year in order to prepare some additional selective characterizations of some Himalayan herbs appeared to have a pronounced protective or healing effect on animals.

-Work package 4 – with minor changes, but all the tasks and scientific objectives were achieved. Only one secondment from the partner DRDO (3 months) was not realized, because of some organizational changes in this partner. The same secondment were transferred to the beneficiary TU in order to achieve the objectives planned.

-Work package 5 – The secondment belonging to the partner DRDO (3 months) was transferred to the beneficiary TU, because of some organizational changes in this partner. The same secondment was transferred to the beneficiary TU in order to achieve the objectives planned.

-Work package 6 – without changes and all the tasks and scientific objectives were achieved. Part of the designed secondments (only 3 months) were not realized and transferred to the other WPs, because the planned objectives on this WP were achieved earlier.

-Work package 7 – this work package was fulfilled in advance and only some secondments (belonging to the partner DRDO – 6 months) were transferred to the beneficiary TU, because of some organizational changes in this partner. The same secondment was transferred to the beneficiary TU in order to achieve the objectives planned.

In regard to the tasks, scientific objectives, deliverables and milestones planned in the project for this reporting period – all of them (and even more) are achieved according to the plan and only some additional publications have to be published on this matter.

The reason for these small amendments, incl. fulfillment of some of the tasks in advance (related to WP7) and the transfer of some other tasks and secondments (related to WP5) from the partner DRDO to the beneficiary TU, is due to some personal and organization changes - some of the researchers included in this project leaved their institutions and we had to find some other researchers to take their place in the planned experimental work. Some health problems of the seconded researchers to DRDO-India also appeared. A replacement of the host institution (from DRDO-India to TU-Bulgaria) for the mice experiment is also done, because of the reconstruction of the vivarium (experimental animal base) in INMAS-DRDO-India. Independently of the circumstance that we fulfilled all the tasks (even some of the tasks were fulfilled in advance) and achieved all deliverables and/or milestones within the time-frame of the planed work, we have still unused several months secondments under this project. We like to use these secondments to develop some new topics and area of common interests (use of nanoparticles along with some new plant extracts, etc) within the same work packages on which we will be able to work with our partners during the next year in order to achieve the best results and to create the most profitable network.

Qualitative indicators of progress and success in line with workplan and milestones (description of progress towards milestones and deliverables)

-WORK PACKAGE 1 - Production of mycotoxins Fumonisin B1 and Ochratoxin A for experimental studies – completely done according to the plan – The produced mycotoxins were used in the planned experiments on the herbal protection against mycotoxins using various kinds of animals/chicks.

-Involved researchers so far: Prof. S. Denev (ER from TU moved to UJ), Prof. S. Stoev (ER from TU moved to UJ), Dr Patrick Njobeh (ER from UJ moved to TU), Ms Judith Phoku (ESR from UJ moved to TU), Prof. M. Dutton (ER from UJ moved to TU), Ms Kh. Ndleve (ESR from UJ moved to

TU)

-Used full-time equivalent months so far – 11.84 months, Planned – 12 months

-Deliverables – completely done:

D1.1. Ensuring of enough quantity of mycotoxin FB1 for experimental work is done

D1.2. Ensuring of enough quantity of mycotoxin OTA for experimental work is done

-Milestone – M1. Production of enough quantity of OTA and FB is done:

-Description of work finished in Work package 1 was done in the previous Periodic Report.

-WORK PACKAGE 2 - Collection of target herbs with known protective, immunostimulating or antimicrobial effect from South Africa and India and receiving some herbal extracts – completely done according to the plan – The collected herbs were used in the planned experimental work on the herbal protection using various kinds of animals/chicks.

-Involved researchers so far: Prof. R. Zheleva (ER from TU moved to DRDO), Prof. V. Gadjeva (ER from TU moved to DRDO), Prof. S. Stoev (ER from TU moved to UJ), Prof. Miroslav Stefanov (ER from TU moved to UJ and DRDO), Prof. Vesselin Ivanov (ER from TU moved to UJ), Ms Ch. Carmen Celia (ESR from UNIKAPOS moved to UJ)

-Involved researchers during the current 2-years Period: Prof. Vesselin Ivanov (ER from TU moved to UJ), Prof. Miroslav Stefanov (ER from TU moved to UJ and DRDO).

-Used full-time equivalent months so far – 11,33 months, Planned – 12 months, Remaining – 0,67 months

Deliverables - done:

D2.1. Ensuring of enough quantity of target herbs for necessary experimental work is done

D2.2. Establishment of biological activity of the same herbs is done

-Milestone – M2. Herb collection is already done, and some additional herbs having immunostimulating or antimicrobial activity were collected and explored during this period:

-Description of work finished in work package 2 - .

Some new indigenous herbs from India with possible activity to reduce the deleterious effects of mycotoxins were collected from different regions with the help of Dr. Rajesh Arora and his team in order to be studied for possible effects on wound granulation or for protective effects on kidneys and liver via the respective studies. A similar collection of herbs was done in S. Africa under the guidance of Prof. Krause (RU), Dr. P. Njobeh and Dr D. Ndinteh (UJ). An additional quantity of the following herbs was collected:

4) Tinospora cordifolia – It is tested for anti-inflammatory, immunostimulating or antioxidative effects and hepatoprotective effect (extract of fresh stems or powder materials were tested)

5) Glycyrrhiza glabra Linn. (Fabaceae). Sanskrit/Indian Name: Yashti-madhu, Yashti-madhuka, Mulhathi, Jethi-madh – it was tested for anti-inflammatory, immunostimulating and hepatoprotective effects.

Fresh stems of Tinospora cordifolia Miers. (Family Menispermaceae) and roots of Glycyrrhiza glabra L. (Family: Fabaceae) were collected from the central plains/plateau region of India with a prevalence of humid subtropical climate, or the comparatively less hotter lower Himalayan tract (250-530 m altitude). The plants were identified at the Institute of Nuclear Medicine and Allied Sciences, Delhi by a qualified Botanist and voucher specimens deposited. The stem portions of Tinospora cordifolia and roots, rhizomes and stolons of were cut into small pieces (ca. 2.5 5-3.0 cm in length) and were shade dried for 72-120 h with the precaution of contamination from the dust. The plant material was further mechanically dried in a hot air oven between 55-60°C for 72 h to remove any left over moisture content. The plant material was then converted into a fine powder form in a grinder and stored in air tight containers preventing it from moisture until the start of the respective experimental work.

-----Staff secondments and transfer of knowledge - Knowledge regarding Identification of some Himalayan and/or South African herb was exchanged between the Indian, South African and

Bulgarian researchers.

WORK PACKAGE 3 - Selective characterisation of some South African and Indian herbs for their bioconstituents (flavanoids, etc) via EPR (Electron Paramagnetic Resonance), NIRS (Near Infrared Reflectance Spectroscopy), NMR, FTIR, UV, EA, MS, RS, MA, etc. in order to explain the mechanism of their antioxidative and protective effects and preparing of target herbal fractions or extracts. In vitro study on antioxidative effect of some herbal extract against oxidative stress via EPR spectroscopy – all necessary work is done

-Involved researchers so far: D. T. Ndinteh (ESR from RU moved to TU and UNIKAPOS), Xavier Siwe Noundou (ER from RU moved to TU and UNIKAPOS), Hilary Ihesinaulo Ezuruike (ESR from RU moved to TU), Bertha Chitambo (ESR from RU moved to TU), G. Beev (ER from TU moved to RU), V. Ivanov (ER from TU moved to RU), I. Dinev (ER from TU moved to UJ), M. Kachleck (ESR from UNIKAPOS moved to UJ), N. Gabor (ER from UNIKAPOS moved to UJ), Manish Adhikari (ESR from DRDO moved to TU)

-Involved researchers during the current 2-years Period: D. T. Ndinteh (ESR from RU moved to UNIKAPOS), Xavier Siwe Noundou (ER from RU moved to UNIKAPOS and TU), Hilary Ihesinaulo Ezuruike (ESR from RU moved to TU), Bertha Chitambo (ESR from RU moved to TU, V. Ivanov (ER from TU moved to RU), I. Dinev (ER from TU moved to UJ), N. Gabor (ER from UNIKAPOS moved to UJ), Manish Adhikari (ESR from DRDO moved to TU)

-Used full-time equivalent months so far – 29,63 months, Planned – 27 months,

Deliverables - done:

D 3.1. Establishing the bioconstituents of some target Himalayan and South African herbs – the work is done according to the plan

D 3.2. Elaboration of technology for preparing some target herbal extracts or fractions and preparing relative publications in this regard. – the work is done according to the plan.

-Milestone – M3. Identification of some herbal bioconstituents and preparation of target herbal extracts or fractions is already done according to the plan

-Description of work finished in work package 3 during the current 2-years Period:

1) Preparation of some herbal extracts or fractions

It was done with the help of all necessary equipment for this e.g., rotary evaporator, lyophilizer etc

-Herbal extracts for selected herbs were prepared with the process of hot maceration. Process has been standardized for preparation of extract(s) from various plant species of the Himalayan region. Selected Herbs from Himalayan region were prepared in various solvent systems i.e., water (100%), water alcohol (50:50); alcohol (100%) targeting specific pre-dominant phytochemicals.

-The yield (%) of the herbs ranged from 10-20%.

-Biofingerprint profile of the selected herbs was done for qualitative analysis of classes of phyto-chemicals presents in the extracts and for obtaining the fingerprints in order to avoid batch to batch variation among herbal fractions.

The table describes the characteristics of the extracts fractionated from the herbs mentioned above. The following extracts were prepared in INMAS, DRDO and the process has been standardized.

-10 plants identified using bioprospection

-5 selected herbs were developed as extracts (range of bioactivity and phyto-chemical fingerprint developed)

2) DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenger antioxidant assay of herbal extracts

The aim of this study was to evaluate how efficient and how much of our samples (six different

extracts of both leaves and stem bark of Erythrina caffra and three marine natural products extracts) is necessary to trap the free radicals.

DPPH● scavenging activity was determined by Sánchez-Moreno's (1998) method, modified into a microplate format. The stable free radical's (DPPH●) purple color in methanolic solution (λmax=517) disappears when it reacts with antioxidants (AH) as in (1) and (2) were described. The product's yellow colour has ignorable absorbance at 517 nm.

DPPH● + AH → DPPH2 + A● (1)

DPPH● + A● → DPPH-A (2)

After half an hour incubation time, the absorbance can be measured with a photometer around λmax. Using different concentrations of the AH, and always the same of DPPH●, EC50 value can be calculated, which means how many antioxidant needs to ignore the half of the free radical.

(_"rem" ^"%")"D" □"PPH" □^"●" "= " □"[DPPH]" □^"●" /□□"[DPPH" □^"●" "]" □_"t=0" "·10" (3)

DPPH● scavenging activity with half-maximal effective concentration (EC50) by using different concentrations of the AH, and always the same of DPPH●, can be calculated. EC50 means how many antioxidant needs to defuse the half of the free radical. An exponential correlation is expected between the remaining DPPH● (3) and the antioxidant concentration, therefore after plotting the ln(%remDPPH●) vs. AH concentration one can read the EC50 value at ln(50) in weight/volume (w/v) terms which can be converted to the more suitable weight/weight (w/w) unit by dividing with the initial concentration of the free radical [DPPH●]t=0. Until the final calculation step everything was expressed as µg/ml which was determined from a calibration curve. Determinations were performed in triplicate and reported on a dry matter. Results are expressed as mean values ± standard deviation.

The results revealed that all of our samples possess antioxidant activity but not with the same efficacy. The medium polar of our samples exhibited the best radical scavenging activity. Some co-publications drafts between Prof Dr Gadjeva, Prof. Stoev, Prof. Dr. Kovacs and Prof Krause groups from Bulgaria, Hungary and South Africa are ongoing for this particular project. Some problems were encountered due to difficulty in using the same conditions for the solubility of the dye and the samples. But after many trials, we managed to overcome that issue by finding the right solvent mixture.

3) Spectrophotometrical analisys of Bulgarian natural and synthetic antioxydants before and after gamma- irradiation – the following tasks were performed:

-Estimation of total polyphenolic content
-Estimation of total flavonoid content
-DPPH radical scavenging activity
-ABTS radical decolourization assay
-Reducing Power Assay
-Linoleic acid degradation assay
-Hydroxyl radical scavenging activity
-Nitric oxide ion scavenging potential
-Antioxidant activity (aqueous phase)
-Protection of membrane against radiation damage (membrane protection index)

4) Cell protection and activation of Bulgarian natural and synthetic antioxydants before and after gamma- irradiation – the following tasks were performed in Indian INMAS lab:

-Radiao-sensitization ability of different natural and synthetic antioxidants, at different doses

-Effects of antioxidants on normal and kidney cells were investigated for toxic/ nontoxic effect at different concentrations. Investigation of radio-sensitization activity was performed.

-Determination of ROS levels in kidney cells. ROS was quantified by monitoring

dichlorofluorescein (DCF) fluorescence in flow cytometer.
-Cell micriscopic analysis.

5) EPR investigations
-Ex vivo assay ascorbate radicals levels, the levels of ROS production, the nitric oxide levels in the tissue homogenates of mice, treated with 80 mg / kg indian natural products by EPR spectroscopy; Biodistribution investigations in organ of 2 Indian antioxidants. Ex vivo of ascorbate radicals levels, the levels of ROS production, the nitric oxide levels in the tissue homogenates of mice, treated with OTA and 80 mg / kg Indian natural products (by EPR spectroscopy; (10 days)
-Direct EPR spectroscopy study on root extracts of Indian antioxydants in powdered and aqueous solution form before and after UV irradiation; In vitro EPR spectroscopy study on DPPH radical scavenging capacity of aqueous root extracts; In vitro spectrophotometry study on DPPH scavenging activity of aqueous root extracts; In vitro EPR spectroscopy study on the effect of incubation time on DPPH radical scavenging capacity of extracts in solution form before and after UV irradiation and gamma irradiation.
Summary
Stable radical 1, 1-dipheny-2-picrylhydrazyl (DPPH) is widely used at in vitro models to investigate antioxidant and radical scavenging abilities of natural extracts. This work presents comparative study on DPPH radical scavenging capacity before and after UV irradiation of aqueous extract of Glycyrrhiza Glabra, a plant species belonging to the Indian flora. DPPH scavenging activities of different extract concentrations (at different incubation time intervals) were analyzed and compared by in vitro spectrophotometry and electron paramagnetic resonance (EPR) spectroscopy. 9.93% and 16.79%, DPPH scavenging activities before and after UV irradiation respectively were found by spectrophotometry. By the EPR spectroscopy study statistical significant increase in DPPH radical scavenging for the Glycyrrhiza Glabra extracts was established after UV irradiation (78.39± 0.001%) comparing to the non irradiated samples (14.02± 0.02).
----Staff secondments and transfer of knowledge
Knowledge regarding Characterization and Extract Preparation of Himalayan and/or South African herb was exchanged between the Indian, South African and Bulgarian researchers using the above described methods/assays.

WORK PACKAGE 4 - Elaboration of mixtures of target herbal extracts for preparing of some sprays/unguents designed for stimulation of wound granulation - is done according to the plan
-Involved researchers so far: M. L. Dlamini (ESR from UJ moved to TU), T. Fonkui (ESR from UJ moved to TU), R. Changwa (ESR from UJ moved to TU), Prerna Agarwal (ESR from DRDO moved to TU), S. Stoev (ER from TU moved to DRDO), Prof. Miroslav Stefanov (ER from TU moved to RU)
-Involved researchers during the current 2-years Period: Prerna Agarwal (ESR from DRDO moved to TU), Prof. Miroslav Stefanov (ER from TU moved to RU)
-Used full-time equivalent months so far – 15,67 months, Planned – 15 months,
Deliverables - done:
D 4.1. Establishing the best mixture of suitable herbal extracts using appropriate constituents, designed for stimulation of wound granulation – is already done.
D 4.2. Elaboration of appropriate unguents or sprays from herbal extracts for testing the stimulation of wound granulation and preparing a research paper in this regard – is done.
-Milestone – M4. Elaboration of herbal sprays/unguents designed for stimulation of wound granulation is already done
-Description of work finished in work package 4 during the current 2-years Period:

(I). Bioactivity linked standardization of extracts targeted for analyzing the stimulating effects on

wound granulations.

10 herbal extracts, as mentioned above in the table were selected for screening on the basis of their antioxidant potential. The primary screening methodology was adopted for screening of herbal extracts based on their free radical scavenging activity (DPPH), modulation capacity of extracts in terms of its ability to act as Superoxide dismutase and reducing potential.

-10-70% reduction of DPPH by selected herbal extracts was observed. The analysis of DPPH scavenging activity of extracts revealed the following order: RDP06> RDP03> RDP09> RDP010> RDP07> RDP08> RDP014> RDP> RDP011.

-SOD like activity of the herbal extracts ranged from 5-35 Units/mg. The analysis of 'SOD like activity' revealed the following order: RDP03> RDP06> RDP09> RDP08> RDP014> RDP07> RDP010> RDP> RDP011.

-ABTS radical scavenging activity of the above mentioned herbal extracts was also analysed. It ranged from 20-80%. The order of scavenging was in accordance with of DPPH radical scavenging activity.

Qualitative and quantitative estimation of the plant constituents known for high antioxidant potential such as flavanoids and polyphenols was done.

-Phenolic content of identified herbals ranges from 80-800 mg/gm of extract, which is 60 to 85% with respect to gallic acid used as standard equivalence

-Flavonoid content of identified herbals ranges from 30 to 70% with quercetin used as standard equivalence. Quantified amount was estimate to be around 25-100 mg/gm of extract.

-The Unit Absorbance Value for these herbals in terms of their total reducing ability was calculated and it ranged from .001- 0.009.

-Correlation analysis with dielectric constant of the solvent systems revealed increase in antioxidant power with respect to decreasing dielectric constant of selected herbal extracts

The extracts RDP-03. 06 and 10 were selected for investigating their stimulating effects on wound granulation.

(II). Various extracts or whole powder from South African herbs Centella asiatica, Withania somnifera, Silybum marianum and Indian herbs Glycyrrhiza glabra, Tinospora cordifolia, Ginger (the rhizome of the Zingiber officinale) and Curcuma Longa (Turmeric) having well defined wound-healing activity and/or antiinflammatory activity and/or antibacterial or antifungal activities were mixed in different proportions with various constituents in order to prepare different unguents, which were tested for improving wound granulation.

WORK PACKAGE 5 - Investigation on stimulating effect of the prepared herbal extracts (unguents or sprays) on wound granulation - finished.

-Involved researchers during the current 2-years Period: G. Terziev (ESR from TU moved to UJ), K. Dimitrov (ESR from TU moved to UJ)

-Used full-time equivalent months – 3,37 months, Planned – 3 months,

Deliverables - done:

D 5. Establishing the best unguents or sprays from herbal extracts for stimulation of wound granulation.

-Milestone – M5 The effect of target herbal extracts (unguents or sprays) as stimulants of wound granulation was already established.

-Description of work finished in work package 4 during the current 2-years Period:

The herb Liquorice (Glycyrrhiza glabra) was found to possess natural antibacterial, antiviral, antifungal, and anti-inflammatory properties in addition to its antioxidative and immunostimulating effects. Some Liquorice constituents, e.g. glycyrrhizin or glycyrrhetic acid possess steroid-like

anti-inflammatory activity, similar to the action of hydrocortisone, which is partially due to inhibition of phospholipase A2 and cyclooxygenase activity, and prostaglandin formation.

The herb Tinospora cordifolia was also found to have similar anti-oxidative, imunostimulating and anti-inflamatory effects and was found to be a suitable herb for wound treatment. The anti-inflammatory effect of this herb was found to be comparable with indomethacin and its mode of action appeared to resemble that of nonsteroidal anti-inflammatory agent and to be effective in both acute and subacute models of inflammation and/or wound granulation - both alcoholic and aqueous extracts of Tinospora cordifolia were found to have such effects being mixed with appropriate constituents. A comparatively potent antibacterial activity of the above herbal extracts were found against some bacterial agents, e.g. Staphylococcus aureus, Proteus vulgaris, Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Pseudomonas aeruginosa, Enterobacter aeruginosa, etc.

Apart from wound healing the herb Centella asiatica was found to combat oxidative damages, e.g. oxidative stress. It was found to have similar anti-inflammatory and anti-bacterial or anti-viral, and immunostimulating properties as the above two herbs.

Some other herbs, which were seen to have a potent anti-inflamatory, anti-oxidative and especially immunostimulating effect for humoral immune response and to be appropriate for using to teat various wounds were found to be Silybum marianum and Withania somnifera.

Target antimicrobial studies:

A) Tetracera potatoria Afzel. Exg. Don (Dilleniaceae) is a medicinal plant used traditionally in Africa for the treatment of tuberculosis related ailments and respiratory infections. The antibacterial activity of the medium polar extracts of T. potatoria leaves and stem bark was recently reported against Mycobacterium smegmatis (MIC 25 µg/mL) and M. aurum (65 µg/mL), two fast-growing Mycobacterium strains used as model micro-organisms for the more pathogenic strain Mycobacterium tuberculosis (Fomogne-Fodjo et al., 2014). The aim of this study was consequently to isolate the compounds possibly contributing to this activity, and which may therefore be promising precursors to be used for the development of novel anti-TB drugs.

Materials and methods: T. potatoria medium polar extract [MeOH/DCM (1:1, v/v)] was fractionated sequentially with petroleum ether to which EtOAC and MeOH were gradually added to increase the polarity. The examination of T. potatoria extract and its fractions was guided by bioassays for anti-mycobacterial activity against M. smegmatis (ATCC 23246) and M. aurum (NCTC 10437) using the minimum inhibitory concentration (MIC) method. All the isolated compounds were structurally elucidated using spectroscopic techniques and evaluated for their anti-mycobacterial activity.

Results: Two novel secondary metabolites (1, 2) named tetraceranoate and N-hydroxy imidate-tetracerane, together with five known compounds [β-stigmasterol (3), stigmast-5-en-3β-yl acetate (4), betulinic acid (5), betulin (6) and lupeol (7)] were isolated and identified. Tetraceranoate exhibited the best activity against M. smegmatis with a minimum inhibitory concentration (MIC) of 7.8 µg/mL, while β-stigmasterol, betulinic acid and betulin showed appreciable anti-mycobacterial activity against both strains (MIC 15 µg/mL).

Conclusion: Seven compounds were isolated from the medium polar extract [MeOH/DCM (1:1, v/v)] of T. potatoria stem bark. Only tetraceranoate one of the isolated compounds showed antibacterial activity against M. smegmatis having efficacy as high as rifampicin (one of a three drug regimen recommended in the initial phase short-course anti-tuberculosis therapy). Thus, tetraceranoate might be an interesting target for systematic testing of anti-TB treatment and management. This research supports the use of T. potatoria in African traditional medicine for the treatment of tuberculosis related symptoms.

B) The leaves, stems and roots of Alchornea cordifolia (Schumach. and Thonn.) Müll. Arg. are used as traditional medicine in many African countries for the management of gastrointestinal, respiratory and urinary tract infections as well as for the treatment of wounds.

Aim of the study: To determine the in vitro antibacterial activity of the crude extracts of leaves and stems of A. cordifolia on gastrointestinal, skin, respiratory and urinary tractpathogens and to identify the compounds in the extracts that may be responsible for this activity.

Materials and methods: The antibacterial activities of crude extracts [hexane, chloroform (CHCl3), ethyl acetate (EtOAc), ethanol (EtOH), methanol (MeOH) and water (H2O)] as well as pure compounds isolated from these extracts were evaluated by means of the micro-dilution assay against four Gram-positive bacteria, i.e. Bacillus cereus ATCC11778, Enterococcus faecalis ATCC 29212, Staphylococcus aureus ATCC 25923 and S. saprophyticus ATCC 15305, as well as four Gram-negative bacterial strains, i.e. Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Moraxella catarrhalis ATCC 23246 and Proteus mirabilis ATCC 43071. The isolation of the active constituents was undertaken by bio-autographic assays in conjunction with chromatographic techniques. The identification and characterization of the isolated compounds were done using mass spectrometry (MS) and Fourier transformed infrared spectrometry (FTIR) as well as 1D- and 2D-nuclear magnetic resonanc e(NMR) analyses.

Results: The leaves and stems of A. cordifolia exhibited varied antibacterial activity against all eight pathogens. Most of the MIC values ranged between 63 and 2000 mg/ml. The highest activities for the crude extracts (63 mg/ml) were observed against S. saprophyticus [stem (EtOAc, CHCl3 and hexane), leaves (MeOH, EtOH, EtOAc and CHCl3)], E. coli [stem (MeOH and EtOH), leaves (MeOH, EtOH, EtOAc and CHCl3)], M. catarrhalis [leaves (EtOAc and CHCl3)], K. pneumoniae [stem (CHCl3), leaves (CHCl3)] and S. aureus [leaves(CHCl3)]. Seven constituents [stigmasterol (1), stigmasta-4,22-dien-3-one (2), friedelin (3), friedelane-3-one-28-al (4), 3-O-acetyl-aleuritolicacid (5), 3-O-acetyl-erythrodiol (6) andmethyl-3,4,5- trihydroxybenzoate(methylgallate) (7)] were isolated from the stem MeOH extract. All these compounds displayed some antibacterial activity against the eight pathogens with highest activity against S. saprophyticus (2 mg/ml). Furthermore, this is the first report of compounds 1, 2, 3, 4, 6 and 7 isolated from A. cordifolia and where a complete set of 2D-NMR data for fridelane-3-one-28-al (4) is presented.

Conclusion: The study demonstrated that the antibacterial activities of A. cordifolia extracts may be due to the presence of the seven isolated compounds, where compounds 3–6 showed the best activity. The observed activity against gastrointestinal, skin, respiratory and urinary tract pathogens supports the traditional use for the treatment of such ailments.

Staff secondments and transfer of knowledge:

The supply with the above target herbs to be used in the study on microbial activity of herbs, e.g. wound granulation in Bulgaria or South Africa and exchange of knowledge on the way of action of these herbs were realized between both countries. Some exchange of knowledge on the stimulating effect of the prepared herbal extracts (unguents or sprays) on wound granulation on rats or farm animals was realized between the researchers from TU, DRDO, RU and UJ via the respective presentations.

WORK PACKAGE 6 – Pig/rabbits experiments (“in vivo” and “in vitro”) on protective effects of herbal additives against toxicity of OTA or/and FB1 – done according to the plan

-Involved researchers so far: Dr Patrick Njobeh (ER from UJ moved to UNIKAPOS), Prof. M. Dutton (ER from UJ moved to UNIKAPOS), M Web (ESR from UJ moved to UNIKAPOS).

-Involved researchers during the current 2-years Period: M Web (ESR from UJ moved to UNIKAPOS).

-Used full-time equivalent months so far – 4,81 months, Planned – 9 months.

-Deliverables:

D 6. Establishing the protective effects of some herbal additives against toxic effects of OTA and/or FB1 in some in vivo and/or in vitro experiments. Preparing a research paper in this regard – the experiment is done and the research paper is under elaboration.

-Milestone – M6. Establishing of possible protective effects of herbal additives against toxic and immunosuppressive effects of OTA and/or FB – the experimental work is done and the publication is currently prepared.

-Description of work finished in Work package 6 was done in the previous Periodic Report. Now a short description of the work (summary of the experiment/results) with rabbits given DON and experimentally protected by herbs is given:

A) The milk thistle Silybum marianum (L). seed extract, called silymarin, has been found to be an useful remedy for liver diseases. Silymarin consists of more than ten identified flavonoids, but the main component is the silibinin, which is a racemic mixture of two diastereomers, silybin A and silybin B. It was proved, that silymarin, as therapeutic agent affects positively the toxin-induced liver damage

The aim of this study was the investigation of any potential positive effect(s) of Carduus marianus on growing rabbits consuming dietary DON in high dosage.

Materials and methods

90 weaned (at the age of 35 days) Pannon White rabbits were housed in metal mesh wired cages (3 rabbits per cage). The rabbits were bred at the farm of Kaposvár University. The experimental trial consisted of two periods. During the first 3 weeks the rabbits were separated in three groups (control, herb 0,5% and herb 1%) and on the 4th week the 3 groups were subdivided into toxin groups as well. Feed was provided ad libitum and the animals had free access to drinking water provided by pacifiers. The animals were checked daily for mortality and morbidity; weight and feed intake were recorded weekly. At the end of the experimental period the rabbits were euthanized by cervical dislocation and were exsanguinated.

DON was produced using Fusarium graminearum strain number IFA 77 (from “Das Interuniversitäre Department für Agrarbiotechnologie”, Tulln, Austria) fungal culture (7 days old), grown on Potato Dextrose Agar (PDA; Chemika-Biochemica, Basil, Switzerland). The homogenized fungal cultures contained DON at concentration of 7140 mg/kg.

Diets were prepared at the Department of Nutrition of Kaposvár University and formulated to meet the nutritional needs of weaned rabbits. Six different batches were prepared: control (C), control with DON (CT), 0,5% of Carduus marianus (H1), 0,5% of Carduus marianus and DON (H1T), 1% of Carduus marianus (H2) and 1% of Carduus marianus and DON (H2T).

Silibinin (SBN) concentration was determined by LC-MS. SBN was used in two concentrations: H1 (71.1±2.5 mg/kg) and H2 (143.6±6.1 mg/kg).

Blood sampling was performed on days 0, 14 and 39 for full blood count, clinical chemistry, immunological parameters’ determination and antioxidant parameters. Blood collection was also performed at the day of slaughtering for antioxidant parameters’ investigation. After the exsanguination, body weight, weight of various organs (liver, kidney, spleen, heart) and caecum were recorded. The pH of the contents of stomach and caecum was measured as well.

Results. Productive performance and slaughtering parameters were not affected significantly by the consumption of DON. The caecal microbiota were affected by an increase in the number of aerobic bacteria when the toxin was consumed (independently of the plant treatment) which is an undesirable incident since in caecum anaerobic conditions occur. C. marianus had no significant effect on antioxidant parameters in rabbits. Despite the high concentration of the toxin rabbits were not affected severly. There was not established any significant interaction of the medicinal plant C. marianus and DON in rabbits.

B) Studies on the cytotoxic effects of extracts of the plants Gunnera perpensa Hydnora abyssinica

and their possible protective effect against mycotoxin T-2

The aim of this study was the investigation of the cytotoxic effect of the extracts derived from the plants Gunnera perpensa and Hydnora abyssinica and subsequently to assess any protective effect against the mycotoxin T-2 using as endpoints cyto- and genotoxicity.

To assess the cytotoxic effect CCK-8 kit was used. CCK-8 is a water tetrazolium salt (derivative of the well-known MTT; Mossman, 1983) which is less toxic than MTT and does not require the additional step of solubilisation. The cells were treated for 48h and five replicates were used for each sample. Five equidistant concentrations of each plant were used (6.25, 12.5, 25, 50 and 100 ug/ml).

Based on the results of the preliminary experiments specific concentrations of each plant extract were chosen to be tested for their possible protective effect against the mycotoxin T-2.

Regarding the extracts derived from Gunnera perpensa there was a clear dose-effect observed whereas in the case of the extracts of Hydnora abyssinica there is a decrease from 6.25 to 25 ug/ml and 50ug/ml but for the 100ug/ml there was an increase (as the student stated this is in accordance with previous antibacterial assays performed at the University of Johannesburg).

Gunnera perpensa viability rates range was 28.5-80.9% whereas for Hydnora abyssinica was 40.3-70.6%.

T-2, the most toxic compound of type A trichothecenes was used at a concentration of 0.5 μM (which corresponds to 0.23 μg/ml) to assess the possible protective effect of the selected extracts. None of the extracts showed any protective effect since the viabilities of the combinations were similar to the viability of the T-2 treated cells.

Genotoxic effects were investigated with the help of Comet assay after exposure to the plant extracts for 24 and 48h. Gunnera perpensa was not genotoxic at all the tested concentrations resulting in a percentage of tail intensity less than 10% (scoring of comets with the Comet IV software). In the case of Hydnora abyssinica regarding the concentrations of 12.5 and 25 ug/ml were not genotoxic but the concentrations of 50 and 100ug/ml there were not enough cells to be scored but from the investigation under the fluorescence microscope it could be concluded that there was a DNA damage corresponding to 2-3 score (estimation-visual scoring).

C) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT assay is a colorimetric assay to assess the metabolic activity of the cells. In the case of our extracts, the objective of the investigation was to evaluate the protective effect of samples (samples from various herbs, e.g. leaves and stem bark of Erythrina caffra) on the lymphocyte cells in the presence of T-2 toxin by cell viability method. The blood was collected from pigs and the lymphocytes were isolated from the blood. The lymphocytes, T-2 toxin and extracts were mixed together and incubated for 24 hours and the MTT was added thereafter, etc.

To assess the cytotoxic effect CCK-8 kit was used. CCK-8 is a water tetrazolium salt (derivative of the well-known MTT; Mossman, 1983) which is less toxic than MTT and does not require the additional step of solubilisation. The cells were treated for 24, 48 and 72h and five replicates were used for each sample. Three different extracts were used-aqueous, methanolic and ethanolic- in three different concentrations (20, 50 and 100 ug/ml).

Based on the results of the preliminary experiments specific concentrations of each plant extract were chosen to be tested for their possible protective effect against the mycotoxin T-2.

Regarding the extracts derived from the leaves ethanolic extract, it was shown to exert the lowest viability in comparison to control with a minimum viability of 52%. In all cases (aqueous, methanolic and ethanolic extract) the highest concentration (i.e. 100 ug/ml) exhibited the lowest viability (dose-effect). A time effect was also observed especially regarding the highest concentration.

In the case of stem derived extracts the aqueous had similar tendency but the methanolic and ethanolic extracts were quite cytotoxic with a cell viability of approximately 20% being observed after 24h.

T-2, the most toxic compound of type A trichothecenes was used at a concentration of 0.5 µM (which corresponds to 0.23 µg/ml) to assess the possible protective effect of the selected extracts. Some extracts didn't show any protective effect since the viabilities of the combinations were similar to the viability of the T-2 treated cells.

Due to the promising results observed in the MTT assay, a comet assay was subsequently performed on the extracts to investigate whether there is a genotoxicity relationship on the mechanism of cells toxicity.

4) Comet assay

The comet assay was performed to evaluate the DNA damage of the lymphocytes cells. In general for this assay a high damage of the DNA is expressed by a large tail of the comet observed. This assay uses almost the same protocol as the MTT (using the same extracts, e.g. E. caffra) where the cells are mixed with the T-2 toxin and the extracts and incubated for overnight. After the incubation, an electrophoresis step was performed to line up the damaged DNA before the analysis on a microscope connected to a computer.

WORK PACKAGE 7 – Chick/mice experiments on protective effects of herbal additives against toxic and immunosuppressive effects of OTA and FB1 – done according to the plan

-Involved researchers so far: Dr Galina Dimitrova Nikolova (ER from TU moved to DRDO), Dr Yanka Dimitrova Karamalakova (ER from TU moved to DRDO), I. Dinev (ER from TU moved to UJ and North-West University)

-Involved researchers during the current 2-years Period: I. Dinev (ER from TU moved to UJ and North-West University)

-Used full-time equivalent months so far – 7,07 months, Planned – 12 months.

-Deliverables:

Establishing the protective effects of some herbal additives against toxic and immuno-suppressive effects of OTA and FB1 in chicks/rats. Preparing a research paper in this regard.– The experimental work is done – some papers and conference reports were done and some others research paper are under elaboration.

-Milestone – M6. Establishing of possible protective effects of herbal additives against toxic and immunosuppressive effects of OTA and/or FB – the experimental work is done and several publications are currently prepared – some of them already published.

-Description of work finished in Work package 7:

A) Development of Nanoformulation from herbal extracts (incl. Silymarin)

SNEDDS (Self NanoEmulsifying Drug Delivery Systems) was prepared to increase the solubility and oral absorption for achieving better bioavailability and therapeutic activity of silymarin. This was attained by dissolving silymarin in oil phase (Labrafac Lipophile WL1349) and then surfactant (Solutol HS 15) and co-surfactant (Transcutol HS) were added as per optimized procedure. It was characterized by determining globule size distribution and Transmission electron microscopy.

Evaluation of efficacy at in vivo level. Various in vivo enzymatic and non-enzymatic experiments e.g., Catalase, Glutathione Reductase, Glutathione Peroxidase and Superoxide dismutase were performed in liver and intestine tissues to reduce mycotoxin-induced toxicity.

Deliverable. Nanoformulation of Silymarin with higher efficacy has been developed.

a) Comparative toxicity analysis with 4 herbal extracts on 4 different mammalian cell lines (HepG2, NKE, ACHN and A498) was performed. Ethanolic Extract of Glycyrrhiza glabra and Tinospora cordifolia at a concentration of 50 µg/ml and 100 µg/ml were found to protect NKE cells, whereas 40-60% cell death was observed in case of transformed cells.

b) Studies on cytotoxicity by Ochratoxin A and Fumonisin B1 were performed on mammalian cell line. Incubation of HepG2 cells with increasing concentration of Ochratoxin A resulted in a dose

and time dependent cytotoxicity as measured by SRB uptake assay. Half lethal concentration (LC50) of OTA was 10 µM and for Fumonisin B1 350 µM after 72 hrs of incubation with OTA.

B) Several ex vivo, in vivo and in vitro experiments were done to evaluate possible protective effects of various herbs against the toxic effects and oxidative stress provoked by ochratoxin A

a) Studies on in-vitro efficacy of herbal extracts against Ochratoxin A

-Cyto-toxic studies with 05 screened extracts against Ochratoxin A on Normal Kidney Epithelial cells (NKE) and tumorigenic Kidney cell line (ACHN) were performed.

-Cyto-toxic studies with 05 Bulgarian extracts with respect to radiation on Normal Kidney Epithelial cells (NKE) was performed in order to validate their efficacy against oxidative stress.

-To validate the antioxidant potential of the extracts, NF-κB activation ability of 6 herbal extracts with radiation was determined on Lac-Z reporter cells. Extract E1 and E2 were found to have highest activity. Studies on changes in cellular physiology (ROS, MMP) with radiation was determined on NKE cells and the results were found to be in corroboration with the above mentioned results.

b) Studies on ex-vivo or in vivo efficacy of herbal extracts [RDP-03, RDP- 06 and; RDP-10] against Ochratoxin A

The studies were performed with the help of EPR in collaboration with Trakia University, Stara Zagora, Bulgaria. 05 herbal extracts (RDP03, RDP06, RDP010, RDP09 and RDP011) were selected for further analysis.

-The presence of free electron in the plant extracts responsible for antioxidant property was determined with the help of EPR.

-The presence of free electron in the plant extract responsible for antioxidant property was determined with the help of g-factor. The g-factor for free electron is ~2.0023. g-factor for different plant extracts was calculated with the help of EPR. The mean deviation for each sample was less than 2%.

-It is verified whether the radical structures registered in the studied samples of RDP 06, RDP03, RDP09, RDP010 and RDP011 extracts belong to a semiquinone free radical; their EPR spectra were evaluated after alkalization.

-The antioxidant capability of the resulting plant extracts against a stable radical DPPH was evaluated and EPR spectroscopy was performed. Plant extracts have shown high efficiency in the DPPH test.

-DPPH free radical scavenging capacities of plant extracts with concentration 1 mg/ml were determined at different time intervals before and after UV irradiation. The results were determined and compared. Statistically significant increase was observed in the DPPH scavenging activity of herbal extracts after 10 min of incubation. Whereas, it started decreasing after 10 min on incubation with DPPH.

-Ex-vivo studies were performed using electron paramagnetic resonance spectroscopy (EPR) methods to elucidate the radical scavenging activity of herbal extracts by following out the levels of some "real time" oxidative stress biomarkers in tissue homogenates and blood sample of experimental animals treated with Ochratoxin A, RDP03 and in combinations of both. It was found that the levels of oxidative stress markers reduced significantly on addition of extract RDP03. In conclusion, we consider further more detailed in vitro and in vivo studies for possible application of those extracts as potential radical scavengers and protectors against different environmental stress causing oxidative damage.

Ex vivo studies with other extracts is in progress.

Animals.

Male white non-inbred mice weighting 25-35 g were used. The mice were housed in

polycarbonate cages in controlled conditions (12 h light/ dark cycles), temperature of 18–23oC and humidity of 40–60%, with free access to tap water and standard laboratory chow.

1 group: The animals (6/per group) were injected by Zingiber officinale, Tinospora cordifolia, Curcuma longa, Glycyrrhiza glabra extract and bacterial sample 006.9 G, 80 mg/kg/ given every day for period of 10 days.

2 group: The animals (6/per group) were injected by Zingiber officinale, Tinospora cordifolia, Curcuma longa, Glycyrrhiza glabra extract and bacterial sample 006.9 G, 80 mg/kg/ given every day + 3 times OTA for period of 10 days. Experiments were carried out in accordance with national regulations and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes. After 10th day the mice were dissected and tissues from liver, kidneys, spleen and heart were homogenates in cold PBS solution and studied by direct and spin trapping EPR spectroscopy and results were compared to those of non treated controls (6 mice).

Electron Paramagnetic Resonance measurements:

Ex vivo assay the levels of ROS production in the tissue homogenates of mice by EPR spectroscopy: Briefly, about 0.1 g of liver, kidneys, pancreas, heart, brain and blood samples were homogenized after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. EPR settings were as follow: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain 1 x 106; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92 s, 5 scans per sample.

Ex vivo assay of the ascorbate radicals levels in the tissue homogenates of mice by EPR spectroscopy: Tissues from liver, kidneys, pancreas, heart, brain and blood n were collected in cold saline and processed immediately. Tissue samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, at 40C for 10 min. Supernatants were collected and the level of Asc. was evaluated by EPR spectroscopy. EPR settings were as follows: center field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1 x 104; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1 scans per sample.

Ex vivo assay of the nitric oxide levels in the tissue homogenates of mice by EPR spectroscopy: Briefly, to 50μM solution of Carboxy PTIO.K dissolved in a mixture of 50 mMTris (pH 7.5) and DMSO in a ratio 9:1. To 100μl tissues of liver, kidneys, pancreas, heart, brain and blood was added 900μl Tris buffer dissolved in DMSO (9:1) after that the mixture was centrifuged at 4000 rpm for 10 min at 4ºC. 100 μL of sample and 100 μL 50 mM solution of Carboxy PTIO were mixed and EPR spectrum of the spin adduct formed between Carboxy PTIO spin trap and generated •NO radicals was recorded. The EPR settings were as follows: 3505 G centerfield, 6.42mW microwave power, 5G modulation amplitude, 75 G sweep widh, 2.5x102 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

Ex vivo assay of the biodistribution levels in the tissue homogenates of mice by EPR spectroscopy:

EPR study on the biodistribution of Curcuma longa, Glycyrrhiza glabra extract 80 mg/kg/ given every day and bacterial sample 006.9 G 40mg / kg and 80 mg/kg/ administratedafter 1hr and 2hrs.

The drug was administrated i.p. and weighing tissue samples of liver, kidneys, pancreas, heart, brain and blood were homogenized in PBS (10% w/v) and centrifuged at 2000 g for 15 min. Supernatants were collected and placed in EPR cavity and EPR spectra of the drug in the different samples were recorded. EPR settings were as follow: center field 3505 G; sweep width 70 G; microwave power 13.02 mW; receiver gain 2 x 104; mod. amplitude 10 G; time constant 327.68 ms; sweep time 327.68 s, 1 scan. The concentration of Psoralea corylifolia Linn. in each sample was determined by double integration of the corresponding EPR spectrum and expressed in arbitrary units.

Results: All of natural compounds acting as a typical antioxidants and reduce ascorbate radicals levels ROS production and nitrite oxide.

Curcuma longa

	Ascorbate radicals	ROS production	NO- radicals
Controls	0.134+-0.00	0.492+-0.01	3.4+-0.74
Liver	0.151+-0.02	0.199+-0.002	0.63+-0.09
Controls	0.43+-0.00	0.369+-0.11	5.48+-0.89
heart	0.013+-0.00	0.278+-0.03	4.77+-0.91

All of natural compounds acting as a typical antioxidants and reduce OTA levels in a combinations and combination OTA+ antioxidant reduce ascorbate radicals, levels ROS production and nitrite oxide.

c) Studies on influence of ochratoxin-A and an extract of Tinospora cordifolia against biochemical and oxidative changes in mice spleen

Collection of the plant material and preparation of Tinospora cordifolia extract:

The stem part of TC for experiment purpose was collected from authenticated Ayurvedic store from a local market at New Delhi, during the month of November, 2013 and was confirmed by expert botanist. Dried stems of TC were cleaned to separate unwanted material and then grinded into coarse powder using mortar and pestle. Fine powder was obtained after grinding in grinder and then passing through sieve. Powdered stems were extracted by Kinetic maceration for 48 hrs using 100% ethanol as solvent. The whole mixture then underwent a coarse filtration by a piece of clean, white muslin cloth which was followed by filtered through whatman no.1 filter paper. The total filtrate was dried till semi-liquid using rotary evaporator (Buchi B-480, India) at 400C and was further lyophilized using lyophilizer (IIshin Lab Co. Ltd, USA) to get the crude extract, and was used as a practical approach to supply the mice with described compounds presumed to protect against OTA intoxication in spleen.

Experimental animals:

Specific pathogen-free male Balb/c mice (second line, non- inbred, weighted 25-35 g mice, 5-6 weeks old) were purchased at two-weeks of age, housed in polycarbonate wire floor cages in controlled conditions (12 h light/ dark cycles), temperature of 18–23oC suitable for their age and humidity of 40–60%, with free access to tap water and standard laboratory chow were maintained. Mice were grouped in 3 experimental groups and 1control group (6 animals in each one/ oral pretreatment/ 11 days experiment) and fed respectively: control group - OTA free, standard diet; group I - TC extract, 80 mg/kg, given 3 times for period of 11 days; group II - 15 ppm OTA, daily in diet; group III – 15 ppm OTA and 80 mg/kg extract of TC. All mice were carefully examined and weighed at 3th, 7th and 11th day (Table 1), and the consumed feed was measured at the end of experiment. For following up the weight and the changes of spleen and internal organs, organs of all treated groups and controls were compared. Experiments were carried out in accordance with national regulations and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes. After 11th day the mice were dissected and spleen tissues were homogenase in cold PBS solution and studied by direct and spin trapping EPR spectroscopy and spectrophotometric biochemical analyses. Results were analyzed and compared to those of non treated controls.

Electron Paramagnetic Resonance measurements:

For all EPR measurements an X-band EMXmicro, EPR spectrometer (Bruker, Germany) equipped with standard Resonator was used. Spectral processing was performed using Bruker WIN-EPR and SimFonia software. The levels of the Asc., NO. radicals and ROS production in experimental mice were calculated by double integration of the corresponding EPR spectra registered in the spleen (arbitrary units).

Ex vivo assay the levels of ROS production in the spleen homogenates of mice by EPR spectroscopy:

The level of ROS productions was studied according to Shi et al., 2005 with some

modifications by Zheleva et al., 2011. Briefly, about 0.1 g of spleen samples were homogenized after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. EPR settings were as follow: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain 1 x 106; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92s, 5 scans per sample.

Ex vivo assay of the ascorbate radicals levels in the spleen tissue homogenates of mice by EPR spectroscopy:

The Asc. levels in organ homogenates were studied according to Buettner & Jurkiewicz, 1993 with slight modifications. Tissues from spleen were collected in cold saline and processed immediately. Tissue samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, at 40C for 10 min. Supernatants were collected and the level of Asc. was evaluated by EPR spectroscopy. EPR settings were as follows: center field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1 x 104; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1 scans per sample.

Ex vivo assay of the nitric oxide levels in the spleen tissue homogenates of mice by EPR spectroscopy:

The levels of •NO radicals were studied according to methods of Yoshioka et al.,1994 and Yokoyama et al.,2004 with some modification. Briefly, to 50μM solution of Carboxy PTIO.K dissolved in a mixture of 50 mMTris (pH 7.5) and DMSO in a ratio 9:1. To 100μl tissues was added 900μl Tris buffer dissolved in DMSO (9:1) after that the mixture was centrifuged at 4000 rpm for 10 min at 4ºC. 100 μL of sample and 100 μL 50 mM solution of Carboxy PTIO were mixed and EPR spectrum of the spin adduct formed between Carboxy PTIO spin trap and generated •NO radicals was recorded. The EPR settings were as follows: 3505 G centerfield, 6.42mW microwave power, 5G modulation amplitude, 75G sweep widh, 2.5x102 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

Ex vivo biochemical analyses of MDA measured specrtophotometrically:

The method estimation of lipid peroxidation of thiobarbituric acid (TBA), which measures Malondialdehyde (MDA)-reactive products, was used (Plaser et al. 1966). In brief, 0.5 mg fresh spleen-tissues, 1 ml physiological solution, and 1 ml 25% trichloroacetic acid were mixed and centrifuged at 7,000 rpm for 20 min. 2 ml protein-free supernatant with 0.5 ml 1% TBA (prepared in 0.025 M NaOH) were added in the reaction mixture. The resultant mixture was then subjected to 95°C for 1 h in a water bath. A pink coloured chromogen complex was formed, readable at 532 nm.

Conclusion

In conclusion, using EPR spectroscopy we have demonstrated increased levels of some “real time” biomarkers of oxidative stress such as Asc., NO. radicals and ROS products in the spleen of mice after treatment by OTA. Moreover combination of OTA with oral administration of TC extract led to significant improvement in the levels of oxidative stress biomarkers in mice spleen.

In the light of these results, TC extract behaves as a good scavenger of ROS and RNS and might find application in further studies in order to find application in the pharmaceutical and food industry as a protector against various mycoses

……d) Studies on protective effect of two essential oils isolated from Rosa damascene Mill. and Lavandula angustifolia Mill, and two classic antioxidants against L-dopa oxidative toxicity induced in healthy mice

Animals

Male non-inbred albino mice (25-40 g) were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), the temperature of 18-23 C and humidity of 40-70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for the protection of animals used for scientific and experimental purposes. Mice were divided into six groups (6 animals in each group). The control group of mice was inoculated two i.p. injections with solvent, only). The second

injection was administered 45 min after the first. To study the Ldopa effect we used the acute model of Bottiglieri et al., 2012. The mice from all tested groups (except controls) received either two i.p. injections of L-dopa (100 mg/kg) followed by benserazide (10 mg/kg). The second injectionwas administered 45 min after the first. The groups undergoing combination therapy were pre-treated first for one hour with i.p. injections in doses of 400 mg/kg of Ascorbic acid, Trolox, Rose oil or Lavender oils according to Umezu et al., 2006 and after that received L-dopa and benserazide. 30 min after the last injection all mice were sacrificed by light anesthesia. Blood was obtained by cardiac puncture and collected in tubes with 10% EDTA (ethylene diaminetetraacetic acid), centrifuged at 3000 rpm for 15 min and plasma samples were carefully separated. The brain was immediately washed in cool saline and was prepared homogenates and centrifuged at 3000 rpm for 15 min. After centrifugation, the samples were immediately studied by EPR spectroscopy for their radical scavenging abilities.

Spectrophotometric methods

All spectrophotometric measurements were performed on a Thermo Scientific spectrophotometer.

Ex vivo spectrophotometric DNPH assay

Quantification of protein carbonyl content (PCC) as final products of protein oxidation was carried out using the spectrophotometric DNPH method described by Dalle-Donne et al. (2003).

Ex vivo spectrophotometry assay for evaluation the levels of MDA

To evaluate the levels of lipid peroxidation, Thiobarbituric Acid Reactive Substances (TBARS) assay was used, which measures MDA reactive substances (Plaser et al., 1966).

Electron paramagnetic resonance (EPR) spectroscopy

EPR measurements of all tested samples were conducted at room temperature (18-23 C) on an X-band EMXmicro, spectrometer Bruker, Germany, equipped with standard Resonator. Quartz capillaries were used as sample tubes. The sample tube was sealed and placed in a standard EPR quartz tube (i.d. 3 mm) which was fixed in the EPR cavity. All EPR experiments were carried out in triplicate and repeated. Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Ex vivo EPR evaluation the levels of +NO radicals

Based on the previously methods (Yoshioka et al., 1996; Yokoyama et al., 2004) we developed and adapted the EPR method for evaluation the levels of +NO radicals. Briefly, the solution of Carboxy. PTIO.K (50 mM) was prepared after dissolving in a mixture of Tris buffer (50 mM, pH 7.5) and DMSO in a ratio 9:1. To 100 ml plasma/brain homogenateswas added 900 ml Tris buffer plus DMSO (9:1) and centrifuged at 4000 rpm for 10 min at 4 C. The tested sample (100 mL) and 100 mL 50 mM solution of Carboxy. PTIO were mixed. The EPR spectrum of the spin adduct formed between the spin trap Carboxy. PTIO and generated +NO radicals was recorded. The levels of +NO radicals were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units. The EPR settings were: 3505 G centerfield, 6.42 mW microwave power, 5 G modulation amplitude, 75 G sweep width, 2.5 x 102 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

Summary of results

Levodopa (L-dopa) is a “gold standard” and most effective symptomatic agent in the Parkinson’s disease (PD) treatment. The several treatments have been developed in an attempt to improve PD treatment, but most patients were still levodopa dependent. The issue of toxicity was raised in vitro studies, and suggests that L-dopa can be toxic to dopaminergic neurons, but it is not yet entirely proven. L-dopa prolonged treatment is associated with motor complications and some limitations. Combining the L-dopa therapy with antioxidants can reduce related side effects and provide symptomatic relief. The natural antioxidants can be isolated from any plant parts such as seeds, leaves, roots, bark, etc., and their extracts riched in phenols can retard the oxidative degradation of the lipids, proteins and DNA. Thus, study suggests that combination of essential oils

(Rose oil and Lavender oil), Vitamin C and Trolox with Ldopa can reduce oxidative toxicity, and may play a key role in ROS/RNS disarm.

e) Studies on ex vivo effect of Glycyrrhiza glabra root extract on some "real time" biomarkers of oxidative stress – an EPR spectroscopy study

Plant extract and Chemicals

The air dried roots of Glycyrrhiza glabra were made into a coarse powder and after dissolved in 2l/ distilled water was subjected to hot maceration process, with continuous stirring for 48h. The water extract was filtered through muslin cloth and the filtrate was concentrated with evaporation on water bath and then lyophilized. The extract was made and providing from INMAS, India as reference. Dimethyl sulfoxide (DMSO), N-tert-butyl-alpha-phenylnitrone (PBN), 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1oxyl-3-oxide (Carboxy-PTIO.K) and and PBS were purchased from Sigma Chemical Co, St. Louis, USA. All the other chemicals used in this study were with analytical grade.

Animals

Male white non-inbred mice weighting 25-35 g were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/ dark cycles), temperature of 18–23oC and humidity of 40–60%, with free access to tap water and standard laboratory chow. The animals (6/per group) were injected by Glycyrrhiza glabra extract, 80 mg/kg/ given 3 times for period of 10 days. Experiments were carried out in accordance with national regulations and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes. After 10th day the mice were dissected and tissues from liver, kidneys, spleen and heart were homogenates in cold PBS solution and studied by direct and spin trapping EPR spectroscopy and results were compared to those of non treated controls (6 mice).

Electron Paramagnetic Resonance measurements

For all EPR measurements an X-band EMXmicro, EPR spectrometer (Bruker, Germany) equipped with standard Resonator was used. Spectral processing was performed using Bruker WIN-EPR and SimFonia software. The levels of the Asc., NO. radicals and ROS production of the plant extract were calculated by double integration of the corresponding EPR spectra registered in the different organs (arbitrary units).

Ex vivo assay the levels of ROS production in the tissue homogenates of mice by EPR spectroscopy: The level of ROS productions was studied according to Shi et al., 2005 with some modifications by Zheleva et al. (2011). Briefly, about 0.1 g of liver, kidneys and pancreas samples were homogenized after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. EPR settings were as follow: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain 1 x 106; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92 s, 5 scans per sample.

Ex vivo assay of the ascorbate radicals levels in the tissue homogenates of mice by EPR spectroscopy: The Asc. levels in organ homogenates were studied according to Buettner & Jurkiewicz, 1993 with slight modifications. Tissues from liver, kidneys, spleen and heart were collected in cold saline and processed immediately. Tissue samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, at 40C for 10 min. Supernatants were collected and the level of Asc. was evaluated by EPR spectroscopy. EPR settings were as follows: center field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1 x 104; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1 scans per sample.

Ex vivo assay of the nitric oxide levels in the tissue homogenates of mice by EPR spectroscopy: The levels of •NO radicals were studied according to methods of Yoshioka et al., and Yokoyama et al., with some modification. Briefly, to 50μM solution of Carboxy PTIO.K dissolved in a mixture of 50 mMTris (pH 7.5) and DMSO in a ratio 9:1. To 100μl tissues was added 900μl Tris buffer dissolved in DMSO (9:1) after that the mixture was centrifuged at 4000 rpm for 10 min at 4ºC. 100

µL of sample and 100 µL 50 mM solution of Carboxy PTIO were mixed and EPR spectrum of the spin adduct formed between Carboxy PTIO spin trap and generated •NO radicals was recorded. The EPR settings were as follows: 3505 G centerfield, 6.42mW microwave power, 5G modulation amplitude, 75 G sweep widh, 2.5x102 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

Summary of results

Glycyrrhiza glabra (Licoric), Indian medicinal plant exhibited good antiinflammatory, antiviral, antimicrobial, antioxidative, anticancer, immunomodulatory, hepatoprotective and cardio- protective properties and excellent ex vivo radical scavenging capacity, which are relevant to radioprotection and uses in medicine. By the present research for the first time using ex vivo Electron Paramagnetic Resonance (EPR) spectroscopy methods to investigate changes in "real time" levels of Ascorbate radicals (Asc.), NO. radicals and ROS production in organs isolated from healthy mice (6 non-inbreed mice) treated with Glycyrrhiza glabra extract (80mg/kg/ given 3 times for 10 days). The mice were dissected and tissues from liver, kidneys, spleen and heart were homogenates in cold PBS solution and studied by direct and spin trapping EPR spectroscopy and results were compared to those of non treated controls (6 mice). Tissue homogenates in DMSO, DMSO solution of the spin trap Carboxy PTIO and DMSO solution of the spin trap N-tert-butyl-alpha-phenylnitrone (PBN) were prepared for determination of the Asc., NO. radicals and for ROS products, correspondingly. It was established that in almost all organs of the treated mice the levels of biomarkers tested were close to those of the untreated controls. Significantly lower levels of nitrite and ascorbate radicals were measured only in the spleens and the hearts of the treated mice compared to controls. Present EPR ex vivo study characterizes Glycyrrhiza glabra water extract as a good antioxidant.

f) Crateva adansonii DC is a plant traditionally used in Cameroon to treat constipation, asthma, snake bites, post menopausal complaints and cancers.

Aim: The anticancer potential of the dichloromethane / methanolextract of C. adansonii stem barks was investigated using human breast cancer cell and 7,12 dimethylbenz(a) anththracene (DMBA)-induced mammary tumorigenesis model in rats.

Material and methods: The cytotoxicity of C. adansonii extract was assessed in vitro towards breast carcinoma (MCF-7 and MDA-MB-231) and non-tumoral cell lines (NIH/3T3 and HUVEC) by Alamar Blue assay. Furthermore, in vivo studies were performed on female Wistar rats treated either with C. adansonii extract at a dose of 75 or 300 mg/kg body weight or with tamoxifen (3.3 mg/kg body weight), starting 1 week prior DMBA treatment and lasted 12 weeks. The investigation focused on tumour burden, tumour DNA fingerprint, morphological, histological, hematological, and biochemical parameters.

Results: CC50 values for the in vitro assays were 289 mg/mL against MCF-7cells and >500 mg/mL in others cells, leading to a selectivity index >1.73. C. adansonii extract significantly ($p<0.001$) revealed in vivo the reduction of the cumulative tumour yield (87.23%), total tumour burden (88.64%), average tumour weight (71.11%) and tumour volume (78.07%) at the dose of 75 mg/kg as compared to DMBA control group. A weak effect was also observed at 30 0mg/kg. This extract showed a moderate hyperplasia at the dose of 75 mg/kg while at 300 mg/kg no significant change was noted as compared to DMBA group. It protected rats from the DNA alteration induced by DMBA and increased antioxidant enzymes activities in mammary gland tissue homogenates. In addition, Ultra-High Performance Liquid Chromatography / ESI-QTOF-Mass Spectrometry analysis of C. adansonii extract detected structure-related of many well-known anticancer agents such as flavane gallate, flavonol, phenylpropanoïds, sesquiterpene derivatives, gallotannins and lignans. The LD50 of C. adansonii was estimated to be greater than 5000mg/kg.

Conclusions: These aforementioned results suggest that the C. adansonii extract may possess antitumor constituents, which could combat breast cancer and prevent chemically-induced breast cancer in rats.

g) Studies on estrogenic potential of Millettia macrophylla. This herb was previously reported to have estrogenic effects and to prevent postmenopausal osteoporosis in Wistar rats. So, the study deals with the identification of its secondary metabolites and the evaluation of their estrogenicity and cytotoxicity toward tumoural cells. Thus, 13 known compounds were obtained from successive chromatographic columns and identified by NMR data compared to those previously reported.

Methods: In vitro estrogenicity of the isolates and the phenolic fraction (PF) of M. macrophylla were performed by E-screen and reporter gene assays, while their cytotoxicity was evaluated by Alamar Blue (resazurin) assay. A 3-days uterotrophic assay and the ability of PF to alleviate hot flushes in ovariectomized adult rats were tested in vivo.

Results: Seven of the 13 secondary metabolites turned to be estrogenic. Only two exhibited cytotoxic effects on MCF-7 and MDA-MB-231 with CC50 values of 110 μM and 160 μM, respectively. PF induced a significant ($p < 0.01$) MCF-7 cells proliferation and transactivated both ERα and ERβ in the reported gene assay at 10−2 μg/mL. In vivo, PF acted more efficiently than the methanol crude extract, resulting to a significant ($p < 0.01$) increase in the uterine wet weight, uterine protein level, uterine and vaginal epithelial height at the dose of 10 mg/kg BW. In addition, PF reduced the average duration and frequency of hot flushes induced in rat.

Conclusion: These aforementioned results indicate that PF is a good candidate for the preparation of an improved traditional medicine able to alleviate some menopausal complaints such as vaginal dryness and hot flushes.

C) Chick experiments were done to evaluate possible protective effects of various herbs against the toxic effects of mycotoxin ochratoxin A.

The experiment with female chicks (broiler ROSS 308) was started on 07/10/2015. Based on the data available with us and also conversion of dose (based on body w. and dose conversion factors available in literature (WHO norms and other standard toxicological manuals) from one animal to another animal/ birds), we have calculated the following dose, which might be the nearest effective dose for alleviating the effects of toxicants, incl. mycotoxins. Some variations and adjustments might be required based on age, sex, breed, and health status of the chicks as well:

I-1. Tinospora cordifolia: 300 mg/kg bw in chicks (oral) – or 4000 ppm via the feed
I-2. Glycyrrhiza glabra: 400-600 mg/kg bw in chicks (oral) - or 6600 ppm via the feed
SA-1. Centella asiatica: 300-400 mg/kg bw in chicks (oral) - or = 4600 ppm via the feed
SA-2. Withania somnifera: 200-400 mg/kg bw in chicks (oral) – or 4000 ppm via the feed
SA-3. Silybum marianum: 80 mg/kg bw in chicks (orally) – or 1100 ppm via the feed

Fresh stems of Tinospora cordifolia Miers. (Family Menispermaceae) and roots of Glycyrrhiza glabra L. (Family: Fabaceae) were collected from the central plains/plateau region of India with a prevalence of humid subtropical climate, or the comparatively less hotter lower Himalayan tract (250-530 m altitude). The plants were identified at the Institute of Nuclear Medicine and Allied Sciences, Delhi by a qualified Botanist and voucher specimens deposited. The stem portions of Tinospora cordifolia and roots, rhizomes and stolons of were cut into small pieces (ca. 2.5 5-3.0 cm in length) and were shade dried for 72-120 h with the precaution of contamination from the dust. The plant material was further mechanically dried in a hot air oven between 55-60°C for 72 h to remove any left over moisture content. The plant material was then converted into a fine powder form in a grinder and stored in air tight containers preventing it from moisture until the start of experiment.

All broiler chicks breed ROSS 308 were fed on Starter feed during the first 10 days (07-16/10/2015). Coli-terravet ® (1g/l) was given to the drinking water during the days 1-3 (07-09/10/2015), EGG BOOSTER (1g/l) was given to the drinking water during the days 4-7 (10-13/10/2015), Bioselet ® E (0,1 ml/l) was given to the drinking water during the days 7-10 (13-16/10/2015). The same scheme/design of medicine was repeated during the next days, etc.

At day 16 (6 days after beginning of experiment - 22/10/2015) SB3 was given in the drinking

water to protect against coccidiosis. All chicks were immunized against Newcastle disease at day 14 (or day 4 after beginning of experiment - 20/10/2015) and reimmunization was done at day 28 (or day 18th after beginning of experiment - 03/11/2015) with vaccine 2510C3U3H

The experiment starts at day 10th after incubation (hatch) of chicks.

11-40 day – all chicks were fed as follow:

-C-1 group - Control 1 - standard grower

-3 ppm group - 3 ppm OTA and standard grower

-5 ppm group - 5 ppm OTA and standard grower

-I-1. Tinospora cordifolia given at 4000 ppm + 3 ppm OTA via the feed

-I-2. Glycyrrhiza glabra given at 6600 ppm + 3 ppm OTA via the feed

-SA-1. Centella asiatica given at 4600 ppm + 5 ppm OTA via the feed

-SA-2. Withania somnifera given at 4000 ppm + 5 ppm OTA via the feed

-SA-3. Silybum marianum given at 1100 ppm + 5 ppm OTA via the feed

Immunization

The control and experimental chicks were immunized at an age of 14 days (4 days after beginning of the experiment) against Newcastle disease with commercial vaccine B1 No 2510C3U3H. A re-immunization was also done at an age of 28 days (18th day after beginning of the experiment). The immunization was realized per os via the drinking water according to the respective prescriptions (2 ml vaccine per 100 chicks).

Assessment of immune response and serological examinations

The blood for serological investigations was taken from the wing vein at an age of 42 days (14 days after re-immunization). The vaccinal immune response was measured by the haemagglutination inhibition test. The viral antigen (strain B1) was used in 8 HU (haemagglutination units). β procedure (Diluted Serum-Constant virus) was performed in 96 well round bottomed microtiter plates. Serial dilutions of the tested serum were made and the antigen was subsequently added. After that, the serum was incubated for 30 minutes and 1% (v/v) chicken erythrocyte suspension was added. The plates were left at room temperature until the known HI- positive wells showed a tight, well-circumscribed button of unagglutinated, sedimented erythrocytes. The haemagglutination inhibiting antibody titer was calculated as the reciprocal value of the highest dilution of serum at which there was complete inhibition of haemagglutination. The results were validated by using negative control serum, which doesn't give a titre >1/8, and a positive control serum with the titre 1: 256 (Hitchner, 1979).

Measurements

The weight of the chicks was measured at day 1 (b.w. was between 40 and 50 g) and at day 10, just before the beginning of the experiment (b.w. was ranged between 190 and 245 g). The chicks were distributed randomly in various experimental or control groups immediately after the last measurement. The quantity of the feed utilized by the chicks in each group, the body weight and the absolute- or relative weight of liver, kidneys, heart, bursa Fabricii, spleen, thymus and carcass were measured at the end of the experiment at day 42 (32 days after beginning of the experiment). Blood/serum for clinical biochemistry and tissues samples for pathomorphological investigations were also taken that time from all experimental and control chicks.

Histological examination

Materials for histological examination were taken from kidneys, liver, lung, heart, spleen, thymus, bursa Fabricii, intestine, brain, cerebellum, medulla and bone marrow. The same were subsequently fixed in 10% neutral buffered formalin or processed for freezing microtome. For proving the fat, the freezing materials were stained with Sudan III. All fixed tissues were subsequently embedded in paraffin, sectioned at 6 µm and stained with haematoxylin-eosin. Periodic acid - Schiff (PAS) staining was also performed for proving of glycoproteid, mucoproteid or lipoproteid substances in various tissues or cell components. Part of the embedded tissues were stained according to Weigert iron haematoxylin to prove the presence or absence of fibrin.

Clinical biochemistry

Various blood and/or serum samples were investigated for various haematological and/or biochemical parameters within 1-2 h of their collection and immediately after separation of the serum. The blood sugar, serum cholesterol and serum creatinine were measured by test of Boehringer Mannheim (Mannheim, Germany); the serum total protein by Bio-La-Test (Lachema Diagnostica, Brno, Czech Republic); the uric acid by EnzUric-FT-test (Labordiagnostica, Gopecke, Germany); the serum albumin according to Kolb and Kamushnikov (1982); the serum enzyme activity of ASAT and ALAT was measured by Cormay test (Smolenskiego, Warsaw, Poland).

Summary of results

Protective effects of herbal feed additives Glycyrrhiza glabra and Tinospora cordifolia against the growth inhibitory effect of ochratoxin A and associated immunosuppression and biochemical or pathomorphological changes were seen. The intensity of pathological and biochemical changes, the changes in relative organs' weight and body weight, and the decrease of antibody titer were strongest in OTA-treated chicks without antidote-supplementation, but the same were less pronounced in chicks treated additionally with Glycyrrhiza glabra and especially in those supplemented with Tinospora cordifolia. The higher relative weight of lymphoid organs of chicks supplemented with both herbs revealed their beneficial effects on immune system. A hepatoprotective effect was seen for both herbs being stronger for chicks additionally supplemented with Glycyrrhiza glabra as can be seen from the pathomorphological findings and the lower levels of ASAT and ALAT. A protective effect on bone marrow and kidneys was found for Tinospora cordifolia, whereas Glycyrrhiza glabra was seen to increase additionally the serum levels of uric acid. It seems, that both herbs could be used as a practical approach for ensuring a better utilization of OTA-contaminated feed as can be seen from the better feed performance and higher body weight of chicks treated with both herbs.

Protective effects of herbal feed additives Silybum marianum or Withania somnifera and slightly of Centella asiatica against the growth inhibitory effect of ochratoxin A and associated immunosuppression and biochemical or pathomorphological changes were seen. The intensity macroscopical and histopathological changes, the deviations in relative organs' weight and body weight, biochemical changes and the decrease of antibody titer were strongest in OTA-exposed chicks without antidote-treatment, followed by chicks treated additionally with Centella asiatica, whereas the same changes were significantly slighter or not seen in chicks additionally treated with the herbal additives Withania somnifera or Silybum marianum. The slighter increase in the serum levels of uric acid and the enzyme activity of ASAT and ALAT also supported the protective effects of the both herbs on the kidneys and/or liver. The strong immunesuppressive effect of OTA on humoral immune response against Newcastle disease was completely prevented in chicks given the herbal additives Withania somnifera or Silybum marianum, which was additionally supported by the higher relative weight of immunocompetent (lymphoid) organs in the same chicks. A hepatoprotective effect was found in OTA-exposed chicks treated additionally with Withania somnifera and Silybum marianum, whereas a nephroprotective effect was only found in chicks additionally treated with Silybum marianum as has been seen from the relative organs’ weight, macroscopic, biochemical and pathomorphological findings. The same herbs or appropriate mixture between them could be used as a practical approach for safely utilizing of OTA-contaminated feed.

Staff secondments and transfer of knowledge

-Bulgarian Researchers exchange some knowledge with Indian and South African researchers regarding animal experimentation.

-Literature regarding mycotoxin and herbal experiments was shared.

-Lecture and presentation on targeted mycotoxins toxicity and herbal protection were delivered by the Bulgarian researchers in the leading Universities in South Africa or India.

-The leads identified have been shared with respective laboratories i.e., Department of Chemistry and Biochemistry, Trakia University, Department of Physiology, Trakia University, Stara Zagora,

Bulgaria, and DRDO-India, and Rhodes University or University of Johannesburg in South Africa, etc.

-Exchange of knowledge between Bulgarian researchers prof. Stefanov and Prof Ivanov and researchers from the Nanotechnology Innovation Centre, under the direction of Prof Nyokong. These include Dr John Mack and Dr Jonathan Britton, Dr Pierre Kempgens on various characterisation techniques, including EPR, NMR, FTIR, UV, Raman, ToF-SIMs, XRD, and XPS.

6. PROJECT ACHIEVEMENTS

Scientific highlights and research achievements:

I. Selective characterization of 10 Indian and South African herbs including herbs from Himalayan region for their bioconstituents profile

• More than 10 Plants originated from Himalayan region or from South Africa, belonging to following families: Fabaceae, Menispermaceae, Zingiberaceae were identified and characterized in order to be screened against targeted mycotoxins (Ochratoxin A and Fumonisin B1). The herbs were selected on the basis of their bioactivity characteristics such as antioxidative, diuretic, protective effects on kidneys and liver, immuno-stimulating or antibacterial effects etc and validated by in silico bioprospection.

• In total 16 herbal extracts were analyzed for the presence of bio-active constituents and antioxidant potential. The bioactivity analysis of selected herbals provided following fingerprints:

 Anti-lipid peroxidation (60-70%)
 Nitric Oxide Scavenging (50-70%)
 Site Specific Hydroxyl Radical Scavenging (70-80%)
 Non-Site Specific Hydroxyl Radical Scavenging (30-40%)

• Qualitative analysis of classes of phyto-chemicals revealed following ranges:

 Alkaloids (Moderate to Extremely High)
 Tannins (Very Low to Low)
 Terpenoids (Moderate to Extremely High)
 Saponins (Low to Moderate)
 Glycosides (Moderate to High)
 Anthraquinones (Very Low to Moderate)
 Proteins (Moderate or otherwise absent)

II. On the basis of the above results 7 herbals were further screened in order to test their efficacy against targeted mycotoxins. Herbals selected belongs to following families:

 Glycyrrhiza glabra (Family: Fabaceae)
 Tinospora cordifolia (Family: Menispermaceae)
 Zingiber officinale (Family: Zingiberaceae)
 Curcuma longa (Family: Zingiberaceae)
 Centella asiatica
 Silybum marianum
 Withania somnifera

III. The antioxidant capability of the resulting plant extracts against a stable radical DPPH was evaluated and the most of plant extracts showed high efficiency in the DPPH test

The following Indian herbs Tinospora cordifolia (in dose 300 mg/kg bw or 4000 ppm via the feed) and Glycyrrhiza glabra (in dose 400-600 mg/kg bw or 6600 ppm via the feed) and following South African herbs: Centella asiatica (in dose 300-400 mg/kg bw or 4600 ppm via the feed), Withania somnifera (in dose 200-400 mg/kg bw or 4000 ppm via the feed) and Silybum marianum (in dose 80 mg/kg bw or 1100 ppm via the feed) appeared to have a good protective effect in broiler chick (breed ROSS) against various toxic effects of mycotoxin ochratoxin A on the body weight, relative organ weight, biochemical indices and humoral immune response. A hepatoprotective effect was seen for Tinospora cordifolia and Glycyrrhiza glabra being stronger for chicks additionally supplemented with Glycyrrhiza glabra as can be seen from the pathomorphological findings and the lower levels of ASAT and ALAT. Protective effects of herbal feed additives Silybum marianum or Withania somnifera and slightly of Centella asiatica against the growth inhibitory effect of ochratoxin A and associated immunosuppression and biochemical or pathomorphological changes were seen, e.g.

protective effects on the kidneys (strongest for Silybum marianum) and/or liver. The strong immunesuppressive effect of OTA on humoral immune response against Newcastle disease was completely prevented in chicks given the herbal additives Withania somnifera or Silybum marianum, which was additionally supported by the higher relative weight of immunocompetent (lymphoid) organs in the same chicks. The same herbs or appropriate mixture between them could be used as a practical approach for safely utilizing of OTA-contaminated feed.

IV. New research in our laboratories has pointed towards the use of natural compounds as universal protectors/mitigators against radiation and mycotoxin toxicity. This property has been exhibited to be present in naturally occurring dietary ingredients, mainly containing flavonoids.

V. Various in vivo or ex vivo enzymatic and non-enzymatic experiments e.g., Catalase, Glutathione Reductase, Glutathione Peroxidase and Superoxide dismutase, etc at liver and intestine tissues revealed the protective effects of some target herbs against mycotoxin-induced toxicity.

VI. The algorithm for assessing radio protective potential of plant extracts and natural products was assessed via some target in vitro and in vivo tests and clinical trials. The mechanisms of radio protective action of the tested extracts and natural products was analysed.

VII. A number of formulation approaches have been employed to increase the solubility and oral absorption of some herbal extracts and products and subsequently to enhance their bioavailability and therapeutic activity.

VIII. Nanoformulation of Silymarin with higher efficacy has been developed. SNEDDS (Self Nanoemulsifying Drug Delivery Systems) was prepared to increase the solubility and oral absorption for achieving better bioavailability and therapeutic activity of Silymarin. The radioprotective efficacy and preliminary mycotoxin toxicity reduction studies revealed that Silymarin nanoemulsion has promising results better than the parent silymarin compound. The silymarin nanoemulsion-pretreated (10µg/ml) irradiated group (Balb/c mice) showed lower frequency of apoptotic bodies and blebbing of human embryonic kidney (HEK) cells as compared to radiation alone group. Survival studies using Balb/c mice confirmed that silymarin exhibits maximum protection at 50 mg/kg b/w against 9 Gy gamma-irradiation. Pre-irradiated treatment with silymarin could restore total lymphocyte counts (TLC) by the 15th day to normal. Based on the series in vivo and in vitro (MTT assay and Annexin V-PI studies, Comet assay and Flow-cytometry) studies, the analysis of data revealed that there is a shift in antioxidant balance upon administration of silymarin that leads to radioprotection. Protection against radiation-induced cell-death and DNA damage by silymarin could be attributed to a reduction in ROS induced by gamma-radiation. In vitro and in vivo experiments showed that silymarin is a promising, effective and safe radiation countermeasure agent and has potential for use during nuclear/radiological emergencies. Our results have clearly shown that the radioprotective efficacy of silymarin nanoformulation is better than silymarin parent compound and preliminary studies indicate its potential ability to reduce mycotoxin-induced toxicity. Therefore, nanosilymarin could be considered as useful source for mitigating both radiation and mycotoxin-induced toxicity warranting further studies to validate its efficacy in in vivo models.

IX. EPR in vitro spectroscopy studies demonstrated that the naturally isolated Piptadenastrium africanum and Haberlea rhodopensis extracts exhibited well expressed DPPH scavenging capacity either before or after UV irradiation. In conclusion, we suggest that further more detailed EPR in vitro and in vivo studies for possible application of those extracts as potential radical scavengers and UV protectors in experimental animal models have to be carried out.

X. The binding ability of ochratoxin A using nano-enabled materials to mitigate exposure was also evaluated. All tested sample materials exhibited strong binding affinity toward OTA in solution. The use of these nanoparticles as feed additives in ameliorating the toxicity of OTA in animals and humans seemed promising. Further studies using some animal models are still required to ascertain the potentials of these materials for use as OTA binders.

XI. Chitosan nanoparticles functionalized with plant extracts for the inhibition of the toxic effects of aflatoxin B1 and ochratoxin A were evaluated (green nanotechnology) with possible applications in

preventing damages caused by these mycotoxins with the aim to improve food safety and boost human and animal health. The chitosan nanoparticles with extracts from medicinal plants (Menta Longifolia and Leonotis leonurus) were synthesised and characterised. The antioxidant ability of extracts was evaluated before being incorporated into chitosan using DPPH radical scavenging assay.
XII. Protective effects of samples from leaves and stem bark of Erythrina caffra were found via MTT assay (cell viability method) on the lymphocyte cells in the presence of T-2 toxin.
XIII. Millettia macrophylla was found to have estrogenic effects and to prevent postmenopausal osteoporosis in Wistar rats. The identification of its secondary metabolites (13 metabolites) and the evaluation of their estrogenicity and cytotoxicity toward tumoural cells was also done.
XIV. The extracts or whole powder from South African herbs Centella asiatica, Withania somnifera, Silybum marianum and Indian herbs Glycyrrhiza glabra, Tinospora cordifolia, Ginger (the rhizome of the Zingiber officinale) and Curcuma Longa (Turmeric) were found to have wound-healing activity and/or antiinflammatory activity and/or antibacterial or antifungal activities in the form unguents or sprays.
XV. The antibacterial activity of the medium polar extracts of T. potatoria leaves and stem bark was found against Mycobacterium smegmatis. The compounds possibly contributing to this activity, and which may therefore be promising precursors to be used for the development of novel anti-TB drugs were established. Seven compounds were isolated from the medium polar extract [MeOH/DCM (1:1, v/v)] of T. potatoria stem bark. Two novel secondary metabolites (1, 2) named tetraceranoate and N-hydroxy imidate-tetracerane were isolated and identified. Tetraceranoate exhibited the best activity against M. smegmatis with a minimum inhibitory concentration (MIC) of 7.8 μg/mL, while β-stigmasterol, betulinic acid and betulin showed appreciable anti-mycobacterial activity against both strains (MIC 15 μg/mL).The isolated compound tetraceranoate showed antibacterial activity against M. smegmatis as high as rifampicin (one of a three drug regimen recommended in the initial phase short-course anti-tuberculosis therapy). Thus, tetraceranoate might be an interesting target for systematic testing of anti-TB treatment and management. This finding supports the use of T. potatoria in African traditional medicine for the treatment of tuberculosis related symptoms.
XVI. The leaves and stems of A. cordifolia exhibited varied antibacterial activity against four Gram-positive bacteria, i.e. Bacillus cereus ATCC11778, Enterococcus faecalis ATCC 29212, Staphylococcus aureus ATCC 25923 and S. saprophyticus ATCC 15305, as well as four Gram-negative bacterial strains, i.e. Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Moraxella catarrhalis ATCC 23246 and Proteus mirabilis ATCC 43071. Seven constituents [stigmasterol (1), stigmasta-4,22-dien-3-one (2), friedelin (3), friedelane-3-one-28-al (4), 3-O-acetyl-aleuritolicacid (5), 3-O-acetyl-erythrodiol (6) andmethyl-3,4,5-trihydroxybenzoate(methylgallate) (7)] were isolated from the stem MeOH extract of A. cordifolia. All these compounds displayed some antibacterial activity against the eight pathogens with highest activity against S. saprophyticus (2 mg/ml). The study demonstrated that the antibacterial activities of A. cordifolia extracts may be due to the presence of the seven isolated compounds, where compounds 3–6 showed the best activity. The observed activity against gastrointestinal, skin, respiratory and urinary tract pathogens supports the traditional use for the treatment of such ailments.
XVII. The investigation of the protective effect of the extracts derived from the plants Gunnera perpensa and Hydnora abyssinica against the mycotoxin T-2 revealed no significant protection.
XVIII. Studies on in-vitro efficacy of herbal extracts against Ochratoxin A using Normal Kidney Epithelial cells (NKE) and tumorigenic Kidney cell line (ACHN) revealed antioxidant potential of the most target extracts. NF-κB activation ability of 6 herbal extracts with radiation was determined on Lac-Z reporter cells and extract E1 and E2 were found to have highest activity.
XIX. Studies on ex-vivo or in vivo efficacy of herbal extracts [RDP03, RDP06, RDP010, RDP09 and RDP011, e.g. Zingiber officinale, Tinospora cordifolia, Curcuma longa, Glycyrrhiza glabra extract] against Ochratoxin A with the help of EPR revealed a high efficiency. It was found that the levels of oxidative stress markers reduced significantly on addition of extract RDP03. In conclusion, we

consider further more detailed in vitro and in vivo studies for possible application of those extracts as potential radical scavengers and protectors against different environmental stress causing oxidative damage.
XX. Studies on influence of ochratoxin-A and an extract of Tinospora cordifolia against biochemical and oxidative changes in mice spleen tissue homogenates using EPR spectroscopy revealed that combination of OTA with oral administration of Tinospora cordifolia extract led to significant improvement in the levels of oxidative stress biomarkers in mice spleen. It seems that Tinospora cordifolia extract behaves as a good scavenger of ROS and RNS and might find application in the pharmaceutical and food industry as a protector against various diseases, e.g. mycotoxicoses.
XXI. Studies on protective effect of two essential oils isolated from Rosa damascene Mill. and Lavandula angustifolia Mill, and two classic antioxidants against L-dopa oxidative toxicity induced in healthy mice revealed that combining the L-dopa therapy (in the Parkinson's disease treatment) with antioxidants can reduce related side effects and provide symptomatic relief. The natural antioxidants can be isolated from any plant parts such as seeds, leaves, roots, bark, etc., and their extracts riched in phenols can retard the oxidative degradation of the lipids, proteins and DNA. Thus, study suggests that combination of essential oils (Rose oil and Lavender oil), Vitamin C and Trolox with L-dopa can reduce oxidative toxicity, and may play a key role in ROS/RNS disarm.
XXII. Studies on ex vivo effect of Glycyrrhiza glabra root extract on some "real time" biomarkers of oxidative stress via EPR spectroscopy revealed that Glycyrrhiza glabra (Licoric) exhibited good anti-inflammatory, antiviral, antimicrobial, antioxidative, anticancer, immunomodulatory, hepatoprotective and cardio- protective properties and excellent ex vivo radical scavenging capacity, which are relevant to radioprotection. It was established that in almost all organs of the treated mice the levels of biomarkers tested were close to those of the untreated controls. Significantly lower levels of nitrite and ascorbate radicals were measured only in the spleens and the hearts of the treated mice compared to controls. This EPR ex vivo study characterizes Glycyrrhiza glabra water extract as a good antioxidant.
XXIII. The anticancer potential of the dichloromethane / methanol extract of Crateva adansonii stem barks was investigated using human breast cancer cell and 7,12 dimethylbenz(a) anthracene (DMBA)-induced mammary tumorigenesis model in rats The results suggest that the C. adansonii extract may possess antitumor constituents, which could combat breast cancer and prevent chemically-induced breast cancer in rats. C. adansonii extract significantly ($p<0.001$) revealed in vivo the reduction of the cumulative tumour yield (87.23%), total tumour burden (88.64%), average tumour weight (71.11%) and tumour volume (78.07%) at the dose of 75 mg/kg as compared to DMBA control group. This extract showed a moderate hyperplasia at the dose of 75 mg/kg while at 300 mg/kg no significant change was noted as compared to DMBA group. It protected rats from the DNA alteration induced by DMBA and increased antioxidant enzymes activities in mammary gland tissue homogenates. In addition, Ultra-High Performance Liquid Chromatography / ESI-QTOF-Mass Spectrometry analysis of C. adansonii extract detected structure-related of many well-known anticancer agents such as flavane gallate, flavonol, phenylpropanoïds, sesquiterpene derivatives, gallotannins and lignans. The LD50 of C. adansonii was estimated to be greater than 5000 mg/kg.

Transfer of knowledge and Training activities (workshops):

Staff secondments and transfer of knowledge in work package 2.

-ER1 moved from TU to DRDO and UJ in order to perform the following objectives:
A) Collection of some new Hymalayan herbs known to have a potent immunostimulating and/or antibacterial effects and receiving some knowledge from Indian and South African scientists in regard to identifying, collecting and preserving some target herbs (leaves, barks, seeds, fruits or

roots) in different stages of their growth known to have strong protective effects on liver and kidneys as well as immunostimulating or antibacterial effects, etc.

B) Exchange of knowledge between Bulgarian, South African and Indian researchers in regards to identifying, collecting and preserving some target herbs in different stages of their growth.

-ER1 moved from TU to RU in order to perform the following objectives:

A) Receiving some knowledge from South African scientists from the south regions of South Africa in regard to identifying, collecting and preserving some target herbs (leaves, barks, seeds, fruits or roots) in different stages of their growth known to have strong protective effects on liver and kidneys as well as immunostimulating or antibacterial effects, etc.

B) Exchange of knowledge between Bulgarian, South African and Indian researchers in regards to identifying, collecting and preserving some target herbs in different stages of their growth.

Staff secondments and transfer of knowledge in work package 3.

-ER1 moved from DRDO (India) to Trakia University (Bulgaria) in order to perform the following objectives:

A) Receiving some knowledge in regard to selective characterization of some Himalayan herbs for their bioconstituents (as the levels of flavonoids, carotenoids, etc) using EPR (Electron Paramagnetic Resonance) and NIRS (Near Infrared Reflectance Spectroscopy) on “Fiber Optic Spectrometer” in order to explain the mechanism of their antioxidative and protective effects on kidneys and liver or their immunostimulating or antibacterial effects and to prepare some extracts or fractions with the aim of exploring their protective abilities.

B) Exchange of knowledge between Bulgarian and Indian ways of preparing various herbal extracts or fractions via mutual presentations & workshops

C) Exploration of in vitro antioxidant properties or ex vivo protective abilities of some herbal extracts or fractions against oxidative stress caused by proper xenobiotics using EPR spectroscopy.

-ESR3 moved from RU (South Africa) to Trakia University (Bulgaria) in order to perform the following objectives:

A) Receiving some knowledge in regard to selective characterization of some South African herbs for their bioconstituents (as the levels of flavonoids, carotenoids, etc) using EPR (Electron Paramagnetic Resonance) and NIRS (Near Infrared Reflectance Spectroscopy) on “Fiber Optic Spectrometer”

B) Exchange of knowledge between Bulgarian and South African ways of selective characterization of herbs and the respective ways of preparing various herbal extracts or fractions via mutual presentations & workshops

C) Exploration of in vitro antioxidant properties or ex vivo protective abilities of some herbal extracts or fractions against oxidative stress caused by proper xenobiotics using EPR spectroscopy.

-ER2 moved from RU (South Africa) to University of Kaposvar (Hungary) in order to perform the following objectives:

A) Transfer of some knowledge in regard to selective characterization of South African herbs for their bioconstituents using some nanotechnologies, NMR, FTIR, UV, EA, MS, RS, MA, etc., and in regard to the way of preparing various herbal extracts or fractions via attending various presentations and workshops.

B) Exchange of knowledge between Hungarian and South African ways of selective characterization of herbs and the respective ways of preparing various herbal extracts or fractions via mutual presentations & workshops

-ER2 moved from Trakia University (Bulgaria) to RU (South Africa) in order to perform the following objectives:

A) Receiving some knowledge in regard to selective characterization of South African herbs for their bioconstituents using some nanotechnologies, NMR, FTIR, UV, EA, MS, RS, MA, etc., and in regard to the way of preparing various herbal extracts or fractions via attending various presentations and workshops.

B) Exchange of knowledge between Bulgarian and South African ways of selective characterization of herbs and the respective ways of preparing various herbal extracts or fractions via mutual presentations & workshops

…..-ER1 moved from University of Kaposvar (Hungary) to University of Johannesburg and Rhodes University (South Africa) in order to perform the following objectives:

A) Receiving some knowledge from South African scientists about selective characterization of some South African herbs or herbal products for their bioconstituents using some nanotechnologies, NMR, FTIR, UV, EA, MS, RS, MA, etc, and in regard to the way of preparing various herbal extracts or fractions via attending various presentations and workshops

B) Exchange of knowledge between Hungarian and South African ways of selective characterization of herbs and the respective ways of preparing various herbal extracts or fractions via mutual presentations & workshops

Staff secondments and transfer of knowledge in work package 4.

-ER1 moved from DRDO (India) to Trakia University (Bulgaria) in order to perform the following objectives: Elaboration of mixtures of target herbal extracts, which will be further tested for their stimulating effects on wound granulation and preparing of some appropriate unguents/sprays using appropriate constituents in this regard via exchanging of some knowledge between Bulgarian and Indian scientists.

-ER1 moved from Trakia University (Bulgaria) to Rhodes University (South Africa) in order to perform the following objectives: Elaboration of appropriate sprays or unguents of mixtures of target herbal extracts, which will be further tested for their stimulating effects on wound granulation and exchanging of some knowledge between Bulgarian and South African scientists.

Exchange of some target knowledge via presentations

Staff secondments and transfer of knowledge in work package 5.

-ESR2 moved from Trakia University (Bulgaria) to University of Johannesburg (South Africa) in order to perform the following objectives: To transfer some knowledge to South African scientists in regard to exploring the stimulating effects of various herbal mixtures on wound granulation in animals at Department of Surgery in Faculty of Veterinary Medicine of Trakia University and to exchange some knowledge between the scientists from both countries.

Exchange of some target knowledge via presentations.

Staff secondments and transfer of knowledge in work package 6.

-ESR1 moved from University of Johannesburg (South Africa) to University of Kaposvar (Hungary) to in order to perform the following objectives:

A) To receive some knowledge from Hungarian scientists in regard to exploring protective effects of some herbs or herbal extracts against toxicity of FB1/DON in rabbits and their possible protective effects on the intestine via the interaction between mycotoxins – herbal extract – gut microbiota.

B) Transfer of some knowledge to South African scientists in regard to protective role of target

plant extracts against the cytotoxic effects of FB1 and/or OTA on pig lymphocyte and/or intestinal cells using various in vitro cytotoxicity tests as MTT assay and Comet assay.

D) Receiving some knowledge about the results of animal experiments produced in University of Kaposvar. Receiving some experience in magnetic resonance imaging and spectroscopy and positron emission tomography when collecting data from live animals as well as their interpretations.

E) Exploring a joint venture with the aim of developing some herbal products based on multiple herbs. Some discussions were consolidated with regards to areas of future joint research.

Staff secondments and transfer of knowledge in work package 7.

-ER2 moved from Trakia University (Bulgaria) to DRDO (India) in order to perform the following objectives: Transfer of some knowledge between Bulgarian and Indian scientists in regard to exploring the protective effects of some herbs or herbal extracts given as feed additives against the OTA-toxicity on various internal organs, the OTA-induced changes in biochemical indices, the OTA-induced decrease in body weight, the OTA-induced immunosuppression and against the oxidative stress provoked by OTA in mice/chicks.

-ER1 moved from Trakia University (Bulgaria) to University of Johannesburg in order to perform the following objectives: Transfer of some knowledge from Bulgarian to South African scientists in regard to exploring the protective effects of some herbs or herbal extracts given as feed additives against the OTA-toxicity on various internal organs, the OTA-induced changes in biochemical indices, the OTA-induced decrease in body weight, the OTA-induced immunosuppression and the oxidative stress provoked by OTA in mice/chicks.

A presentation/workshop was given/attended by Bulgarian scientists in this regard.

THE TRANSFER OF KNOWLEDGE AND TRAINING ACTIVITIES (WORKSHOPS) WERE DONE IN VARIOUS WORK PACKAGES VIA THE FOLLOWING ACTIVITIES:

- Screening of Herbal extracts for their anti-toxin efficacy. Toxicological studies were performed on both normal and transformed cells in relation to efficacy of herbal extracts.
- 14 more extracts of herbal materials were studied for their efficacy.
- Standardization of bioassay protocols to evaluate nutraceutical standardization; antioxidant activity in both lipid and aqueous phase, free radical induced flux and; ex vivo systems for anti-lipid per-oxidation potential. These assays are used to standardize the nutraceuticals for its efficacy which reduces with time (due to varied storage conditions). Such assays were carried out jointly and necessary training imparted.
- In silico biprospection model: A standardized mathematical model developed in house at the laboratory has been shared and necessary training imparted to use this model for selection of nutraceuticals based of multi-parametric based matrix analysis.
- Process standardized for herbal preparation preventing loss of thermolabile compounds was shared and jointly performed for development of multiple solvent-system based nutraceuticals.
- The extraction of plant materials and compound isolation in Rhodes University (South Africa) was carried out with participation of visiting Marie Curie fellows by using various chromatographic techniques including low pressure column chromatography, preparative thin layer chromatography, high pressure liquid chromatography, high speed counter current chromatograhopy.
- The extraction and characterization of plant materials and compound isolation in Trakia University (Bulgaria) was carried out with participation of visiting Marie Curie fellows by using EPR (Electron Paramagnetic Resonance) and NIRS (Near Infrared Reflectance Spectroscopy) on “Fiber Optic Spectrometer”, etc.
- Training course of Hungarian Marie Curie fellow Nagy Gabor in the Department of Biotechnology and Food Technology at Faculty of Science in University of Johannesburg (UJ) and

participation in some research within the frame of Marie Curie IRSES project. Acquire skills in the extraction of active medicinal plant components and characterizing them using various chromatographic techniques including TLC and GC-MS/MS under the guidance of, Dr Nginteh Derick, MTT assay and Comet assay.

- Participation of Bulgarian fellows Miroslav Stefanov and Vesselin Ivanov in training activities in regard to characterization of plant metabolites in RU using nuclear magnetic resonance (NMR), Fourier Transform Infrared Spectroscopy (FTIR), ultraviolet (UV), elemental analyses (EA), Mass Spectroscopy (MS), Raman spectroscopy (RS), Mossbauer analyses (MA), etc.
- Training course of South African Marie Curie ESR fellow Hilary Ihesinaulo Ezuruike, Xavier Noundou, Derek Ndinteh and Bertha Chitambo in the Dept of Chemistry, Faculty of Medicine, Trakia University and participation in some research within the frame of Marie Curie IRSES project via using EPR (Electron Paramagnetic Resonance) or NIRS (Near Infrared Reflectance Spectroscopy), etc.
- Training course of Indian Marie Curie ESR fellows Prerna Agarwal and Manish Adhikari in the Dept of Chemistry, Faculty of Medicine, Trakia University and participation in some research within the frame of Marie Curie IRSES project via using EPR (Electron Paramagnetic Resonance) or NIRS (Near Infrared Reflectance Spectroscopy), etc.
- Participation of Indian Marie Curie ESR fellows in animal experiments with mice in Trakia University in Bulgaria.
- Training courses or specializations in different areas of research organized by various participants in different countries for receiving target skills.
- Participation in various in vitro or in vivo experiments and exchange of knowledge or receiving some experience in various technics such as magnetic resonance imaging and spectroscopy, positron emission tomography, MTT assay, EPR (Electron Paramagnetic Resonance), NIRS (Near Infrared Reflectance Spectroscopy), DPPH radical scavenging assay, ABTS diamonium salt radical cation decolorization test is also used as a radical scavenging test, Comet assay, Annexin V-PI (propidium iodide) studies, flow-cytometry, etc.
- Specialization of Bulgarian Marie Curie ESR fellows Miroslav Stefanov, Vesselin Ivanov, Georgi Terziev and Ivan Dinev in the Department of Biotechnology and Food Technology, Faculty of Science at the University of Johannesburg (UJ) within the frame of Marie Curie IRSES project and acquiring some additional skills in chromatographic instrumentations that include HPLC, TLC, LC-MS and GC-MS under the guidance of Dr PB Njobeh and Dr Dereck Ndinteh.

Work of master students from UJ involved in our IRSES project:

In the University of Johannesburg, there are several students projects within the frame of the Marie Curie IRSES staff exchange programme HERBAL PROTECTION with several postgraduate and academic staff members within the Department of Biotechnology and Food Technology and the Department of Applied Chemistry, which are involved in this IRSES programme. Some of these projects are completed that have led to awards of Master's degree and also generated research outputs, while others are in the final stages of completion. Some of these are outlined below:

1) PhD project by the Marie Curie IRSES fellow Fonkui Youmbi Thierry, "Mitigating the occurrence of mycotoxins and their effects thereof using nano-enabled binders.", Supervisor Dr Patrick Njobeh and Co-supervisor Prof R. Krause (all of them participated in our IRSES project)

2) Masters student project by the Marie Curie IRSES fellow in the new MSC (Nanoscience Programme), Kulani J, "Chitosan nano-particles functionalized with protective plant extracts for the inhibition of aflatoxin B1 and Ochratoxin A activity", Supervisor Dr Patrick Njobeh and Co-supervisors Prof. A. Mishra and Prof Rui Krause (some involved in our IRSES project).

3) Masters student project by the Marie Curie IRSES fellow Dlamini ML, "The application of some target formulations of active herbal plant components in reducing animal exposure to mycotoxins and their possible effects", Supervisor Dr Patrick Njobeh and Co-supervisor Prof R.

Krause (all of them participated in our IRSES project).

4) Masters student project by the Marie Curie IRSES fellow Khanyisa Ndleve, “Mycotoxin dietary exposure levels in humans in the rural areas of KZN”, Supervisor Dr Patrick Njobeh, participant in our IRSES project.

5) Masters student project by the Marie Curie IRSES fellow Mr Veli Thipe Clement, “Optimization of the antifungal activity of several antifungal agents using gold nanoparticles (AuNPs) synthesized through green chemistry.” Supervisor Dr Patrick Njobeh and Co-supervisor Prof Sabelo Mhlanga (participated in our IRSES project).

Current Projects of students from Rhodes university partly connected with our IRSES project

1) Synthesis of iron nanoparticles coated with plant exudates for mycotoxin concentration and extraction.

2) Coated nanoparticles for mycotoxin destruction in food and feed

3) Coated Nanoparticles for pre-concentration of secondary metabolites from marine bacteria and fungi

Two of these projects are developing at the postdoc level and the other as an Honours project (4th-year BSc)

Some of the mentioned above students and/or supervisors travelled to Bulgaria or Hungary for some training and exchange of knowledge.

Dissemination of results (conferences, publications...):

PRESENTATIONS OF OUR IRSES PROJECT AND PARTICIPATION IN WORKSHOPS AND SEMINARS:

• 1) 06/02/2015 – Briefing – press center – Stara Zagora. Presentation of Marie Curie IRSES project 316067 “HERBAL PROTECTION” in the mass media via press conference given by Coordinator of the project Prof. S. Stoev and the Bulgarian coordinator Prof V. Gadjeva (representative of Trakia University) and Dr Manish Adhikari – representative of DRDO, INMAS-India
• 2) 25 September 2015 – Marie Curie Researcher Night Workshop, – Prof. Stoycho Stoev – project coordinator, Presentation of the Marie Curie IRSES (International Research Staff Exchange Scheme) project 316067 “HERBAL PROTECTION”: “Studies on some herbal additives giving partial protection against toxic or immunosuppressive effects of some mycotoxins” (2013-2016)
• 3) 09 November 2015 - Invited lectures/workshops at International Conference on „New Challenges in Mycotoxin Research”on the topics: “New challenges related to animal health aspects of mycotoxins –project coordinator prof. S. Stoev” and “New challenges related to humanl health aspects of mycotoxins – project participant Prof. M. Dutton” in University of Kaposvar in Hungary
• 4) 10th December, 2016 – Presentation of IRSES project via presentation on “Impact of climate change on mycotoxins in food: management interventions by herbs of Indian, European & South African origin" by Dr Rajesh Arora from DRDO in National Seminar on “Challenges of Climate Change and Green Environmental Solutions” organized by Chaudhary Charan Singh University, Meerut, India.

THE PRINCIPAL PUBLICATIONS IN AUTHORITATIVE PEER-REVIEWED INTERNATIONAL JOURNALS AND CONFERENCES/POSTERS RESULTING FROM THE PROJECT:

• 1) Nikolova G, Karamalakova Y, Kovacheva N, Stanev S, Zheleva A, Gadjeva V, Protective effect

of two essential oils isolated from Rosa damascene Mill and Lavandula angustifolia Mill, and two classic antioxidants against L-dopa oxidative toxicity induced in healthy mice, Regulatory Toxicology and Pharmacology 81 (2016) 1-7. IF=2.22
• 2) Agarwal P, Karamalakova YD, Adhikari M, Gupta D, Nikolova GD, Hadzhibozheva PV, Gadjeva VG, Stoev S, Arora R, Zheleva A, Investigations on DPPH scavenging capacity before and after UV-irradiation of aqueous root extract of Glycyrrhiza Glabra, ISSN: 1314-6246, J. BioSci. Biotechnol., 2015, SE/ONLINE: 183-188.
• 3) Agarwal P, M Adhikari, G Nikolova, Dr Gupta, T Georgiev, V Gadjeva, S Stoev, R Arora, Y Karamalakova and A Zheleva, Ex vivo effect of Glycyrrhiza Glabra root extract on some "real time" biomarkers of oxidative stress – an EPR spectroscopy study, J. BioSci. Biotechnol.
• 4) Karamalakova Y, P Agarwal, G Nikolova, M Adhikari, D Gupta, S Stoev, T Georgiev, P Hadzhibozheva, R Arora, Z Zhelev, S. Raisuddin, V Gadjeva and A Zheleva, Influence of ochratoxin-A and an extract of Tinospora cordifolia against biochemical and oxidative changes in mice spleen, Science & Technologies, Medical Biology Studies, Clinical Studies, Social Medicine And Health Care, Volume 6 (1), 2016, 242-251.
• 5) Karamalakova Y, Stoev S, Gadjeva V, Nikolova G, Indian ayurvedic plants with potentially protective activities against ochratoxin A induced-toxicity, Proceeding of reports from annual university scientific conference, 20-21 October, 2016. Veliko Turnovo, ISSN 2367-7481.
• 6) Stoev SD, K. Dimitrov, N Grozeva, I. Zarkov, T. Mircheva, D. Zapryanova, I. Valchev, S. Denev, S. Chobanova, M. Stefanov, P.B. Njobeh, Some herbal feed additives giving partial protection against ochratoxin A toxicosis in broiler chicks, In press
• 7) Stoev SD, I. Zarkov, T. Mircheva, D. Zapryanova, S. Denev, R. Arora, Some Indian herbs having protective effects against deleterious effects of ochratoxin A in broiler chicks, In press
• 8) Fomogne-Fodjo MCY, DT Ndinteh, DK Olivier, P Kempgens, S van Vuuren, RWM Krause, Secondary metabolites from Tetracera potatoria stem bark with anti-mycobacterial activity, Journal of Ethnopharmacology, 195 (2017) 238–245, DOI: http://dx.doi.org/10.1016/j.jep.2016.11.027. IF=3.05
• 9) Noundou X.S., R.W.M. Krause, S.F. van Vuuren, D. T. Ndinteh, D.K. Olivier, Antibacterial effects of Alchornea cordifolia (Schumach. and Thonn.) Müll. Arg extracts and compounds on gastrointestinal, skin, respiratory and urinary tract pathogens, Journal of Ethnopharmacology, 179 (2016) 76–82. IF=3.05
• 10) Zingue S, J Tchoumtchoua, DM Ntsa, LP Sandjo, J Cisilotto, CBM Nde, E Winter, CF Awounfack, D Tantoh Ndinteh, C Clyne, D Njamen, M Halabalaki, TB Creczynski-Pasa, Estrogenic and cytotoxic potentials of compounds isolated from Millettia macrophylla Benth (Fabaceae): towards a better understanding of its underlying mechanisms, BMC Complementary and Alternative Medicine (2016) 16:421, DOI 10.1186/s12906-016-1385-5. IF=1.98
• 11) Zingue S, J Cisilotto, AB Tueche, A Bishayee, FA Mefegue, LP Sandjo, CBM Nde, E Winter, T Michel, D Tantoh Ndinteh, CF Awounfack, KK Silihe, TTM Tanekou, TB Creczynski-Pasa, D Njamen, Crateva adansonii DC, an African ethnomedicinal plant, exerts cytotoxicity in vitro and prevents experimental mammary tumorigenesis in vivo, Journal of Ethnopharmacology 190 (2016) 183–199. IF=3.05
• 12) Agarwal P, Yanka Karamalakova, Manish Adhikari, Damodar Gupta, Galina Nikolova, Raman Chawlal, Veselina Gadjeva, Stoycho Stoev, Rajesh Arora, and Antoaneta Zheleva, Aqueous root extract of Glycyrrhiza Glabra: An comparative study of the reaction with DPPH, National Conference of Young Researchers "Biological Science for better future", 30-31 October, 2015, University of Plovdiv, Biological Faculty, Plovdiv, pp 48-49
• 13) Agarwal P, Rajesh Arora, Manish Adhikari, Damodar Gupta, Galina Nikolova, Raman Chawlal, Veselina Gadjeva, Stoycho Stoev, Yanka Karamalakova and Antoaneta Zheleva, Glycyrrhiza Glabra: "Real Time" oxidative status of animals, National Conference of Young Researchers "Biological Science for better future", 30-31 October, 2015, University of Plovdiv,

Biological Faculty, Plovdiv, pp 52-53.
• 14) Adhikari M, Karamalakova Y, Nikolova G, Gupta D, Chawia R, Ivanov V, Kumar R, Zheleva A, Gadjeva V, Arora R, Stoev S, Nanosilymarin as an antioxidant agent: Comparative in vitro studies. XIV International Congress of Medical Sciences, Sofia, Bulgaria, 7-10 May, 2015, Suppl 1, pp 93.
• 15) Arora R, D. Gupta, R. Chawla, P. Agarwal, M. Adhikari, Yana Karamalakova, G. Nikolova, V. Ivanov, M. Stefanov, M. Kovács, V. Gadjeva, Stoycho Stoev, Impact of climate change on mycotoxins in food: management interventions by herbs of Indian, European & South African origin, National Seminar on "Challenges of Climate Change and Green Environmental Solutions". 10th December, 2016. Organized by Chaudhary Charan Singh University, Meerut, India, pp 37-38.
• 16) Karamalakova Y, S. Stoev, V Gadjeva, G Nikolova, Indian Ayurvedic Plants with Potentially Protective Activities against Ochratoxin A induced toxicity, Annual University Scientific Conference, 20-21 October, 2016, Military University, Vassil Levski, Veliko Turnovo, pp 26.
• 17) Agarwal P, G. Nikolova, M. Adhikari, D. Gupta, S. Stoev, T Georgiev, P. Hadzhibozheva, V. Gadjeva, R. Arora, A. Zheleva, Y. Karamalakova, Effect of Tinospora cordifolia on Ochratoxin A-induced oxidative stress in mice spleen: Electron Paramagnetic Resonance and Biochemical Study, XXVI International Scientific Conference, 2-3 June, 2016, pp 35.
• 18) Agarwal P, Nikolova G, Adhikari M, Arora R, Stoev S, Zheleva A, Gadjeva V, Karamalakova Y, Ochratoxin A: Effects of plant antioxidants on metabolic oxidative transformation and nephrotoxicity in mice. XV International Congress of Medical Sciences, Sofia, Bulgaria, 12-15 May, 2016, Suppl 1, pp 86.
• 19) Karamalakova Y, Prerna Agarwal, Galina Nikolova, Manish Adhikari, Petia Hadzhibozheva, Tsvetelin Georgiev, Damodar Gupta, Protective effect of Tinospora cordifolia against ochratoxin A-induced oxidative stress in mice, Competition "Science and Youth", Auditorium complex Plovdiv, 12-14 May, 2016.
• 20) Karamalakova Y, Prerna Agarwal, Galina Nikolova, Manish Adhikari, Petia Hadzhibozheva, Tsvetelin Georgiev, Damodar Gupta, Cardioprotective activity of extract from Tinospora cordifolia against chronic ochratoxin A induced oxidative dysfunctions, Competition "Science and Youth", Auditorium complex Plovdiv, 12-14 May, 2016, pp 17.
• 21) Stoev, S.D. (2015) New challenges related to animal health aspects of mycotoxins, International Conference on „New Challenges in Mycotoxin Research, 9 November, 2015, University of Kaposvar, Hungary
• 22) Yanka Karamalakova, Raj Kumar; Rakesh K. Sharma; Rajesh Arora, Galina Nikolova, Ekaterina Georgieva, Antoaneta Zheleva, Veselina Gadjeva, Investigation the influence of natural antioxidants on "Real Time" oxidative status of animals, 70 years anniversary Scientific conference, 30-31 October, 2015, University of Plovdiv, Biological Faculty, Plovdiv.
• 23) Adhikari M., Arora R., Karamalakova Y., Kumar R., Ivanov V., Zheleva A., Gadjeva V., Stoev S. Gamma radiation reduced DNA damage attenuation by nano-silymarin: an in vitro approach. Folia medica, 2015, 57, Suppl 1, 62, Medical University, Plovdiv, Bulgaria
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• 25) Manish Adhikari, Yana Karamalakova, Veselin Ivanov, Damodar Gupta et al. Evaluation of Silymarin as a Prospective Countermeasure against Radiation and Mycotoxin-induced Toxicity. Medical conference, Stara Zagora, Bulgaria, Poster (2015).
• 26) C C Celia, M L Kachlek, Zs Gerencsér, Zs Matics, Zs Szendrő, A Dalle Zotte, V Giaccone, M Kovács: Effect of Carduus marianum herb on the productive performances of growing rabbits. In: Steffen Hoy (szerk.), 19. Internationale Tagung über Haltung und Krankheiten der Kaninchen, Pelztiere und Heimtiere [19th International Symposium on housing and diseases of rabbits,

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• 27) Kachlek M, Szabó-Fodor J, Bonai A, Bors I, Celia C, Gerencsér Zs, Matics Zs, Szendrő Zs, Dalle Zotte A, Kovács M: Assessing the possible interaction between Carduus Marianus and dietary deoxynivalenol on caecal microbiota and fermentation of growing rabbits. Poljoprivreda (osijek) 21:(Supplement 1) pp. 186-189 (2015)
• 28) Siwe Noundou X., Chitambo B., Bors I., Zsuzsanna H.K., Zsófia B.B., Kachlek M., Krause R.W.M., van Vuuren S. and Kovács M. Erythrina caffra: A broad spectrum of biological activities. Poster presentation, 42nd National Convention of the South African Chemical Institute (SACI), 29th November –4th December 2015, Durban, South Africa.
• 29) Vergina Mateva, Yanka Karamalakova, Prerna Agarwal, Manish Adhikari, Galina Nikolova. Immuno-modulatory Effects of Curcuma longa L. Extract Against Ochratoxin (A) - Induced Reactive Oxygen Species and Immune Response. Poster presentation; Leiden International (bio-) Medical Student Conference 2017, Leiden , The Niderlands (in press)
• 30) Stoev, S. D. Foodborne mycotoxicoses, risk assessment and underestimated hazard of masked mycotoxins and joint mycotoxin effects or interaction, Environmental Toxicology and Pharmacology, 2015, 9, 794–809. (http://dx.doi.org/10.1016/j.etap.2015.01.022) IF=2,09
• 31) Stoev, S. D. Balkan Endemic Nephropathy – Still continuing enigma, risk assessment and underestimated hazard of joint mycotoxin exposure of animals or humans, Chemico-Biological Interactions, 261 (2017) 63-79, doi: 10.1016/j.cbi.2016.11.018 (http://dx.doi.org/10.1016/j.cbi.2016.11.018) IF=2.93
• 32) Motaung L, Yah C, Mavumengwana V, Abia WA, Sekhejane P, Njobeh PB, 2015 South African rural population perception on mycotoxin contamination of food commodities and associated health implications. Oral presentation at the 9th Annual Africa Young Graduates and Scholars Conference, Cape Town, South Africa, 30 March – 01 April, 2015.
• 33) Motaung L, Yah C, Mavumengwana V, Abia WA, Sekhejane P, Njobeh PB, 2015 Mycotoxin contamination of South African agricultural commodities and their threats on food safety and social wellbeing. Poster presentation at the 9th Annual Africa Young Graduates and Scholars Conference, Cape Town, South Africa, 30 March-01 April, 2015.
• 34) Thierry F Youmbi, Vuyo Mavumengwana, Rui W Krause, Patrick B Njobeh, 2015 Silica nanoparticles and silicate derivatives as ochratoxin A binding agents. Oral presentation at the 6th International Conference of Food Factors (ICoFF) conference, South Korea, Nov 22-25, 2015
• 35) Njobeh PB, 2015 Current trends of mycotoxin contamination in foods and feeds, analytical and management challenges from the African perspective. Plenary lecture at the 4th Cameroon Society of Toxicological Sciences (CSTS) conference, Yaounde, Cameroon, 26-29 May 2015.
• 36) Khoza BS, Gbashi S, Steenkamp PA, Njobeh PB, Madala NE, 2016. Identification of hydroxylcinnamoyl tartaric acid esters in Bidens pilosa by UPLC-tandem mass spectrometry. South African Journal of Botany. 103, 95-100. IF=1.24.

MONOGRAPHS, CHAPTERS IN BOOKS

• 37) Stoev, S. D., Endemic Mycotoxic Nephropathies in farm animals and humans – complex aetiology, diagnostics, prophylaxis, hygiene control and risk evaluation (Subtitle: Complex aetiology of Endemic Nephropathy and possible prophylaxis), LAP LAMBERT Academic Publishing, Saarbrücken, Germany, ISBN 978-3-659-74828-8, 2015, pp. 1-154.
• 38) Stoev, S. D., Food Security and Foodborne Mycotoxicoses, Risk Assessment, Preventive Measures, and Underestimated Hazard of Masked Mycotoxins or Joint Mycotoxin Interaction, In: Food Toxicology, Chapter 9, Debasis Bagchi, Anand Swaroop (Eds), CRC Press, Taylor & Francis Group, 2016, ISBN 9781498708746, pp 169-199

7. PROJECT MANAGEMENT

Overview of the activities carried out by the partnership; Identification of problems encountered and corrective action taken:

REALIZED VISITS AND MEETINGS AND CONFERENCES/WORKSHOPS RELATED TO IRSES PROJECT

• 1) Organizing of press conference given in the Breefing Press Center in Stara Zagora on 06th of February 2015 for Presentation of achievements of Marie Curie IRSES project 316067 "HERBAL PROTECTION". Various achievements and profits derived from this project were presented in the mass media via by the coordinator of the project Prof. S. Stoev and the Bulgarian coordinator Prof V. Gadjeva - representative of Trakia University and Dr Manish Adhikari – representative of DRDO, INMAS - India.
• 2) Participating in Marie Curie Researcher Night Workshop on 25 September 2015 and presentation of project achievements by the coordinator Prof. Stoycho Stoev, incl. poster presentation of the Marie Curie IRSES (International Research Staff Exchange Scheme) project 316067 "HERBAL PROTECTION" on the topic: "Studies on some herbal additives giving partial protection against toxic or immunosuppressive effects of some mycotoxins" (2013-2016)
• 3) Organizing of International Conference "New Challenges in Mycotoxin Research" and Workshop by Prof. Melinda Kovac (beneficiary 2) at University of Kaposvar on 09th of November 2015. Invited lectures in the International Conference were given by Prof. S. Stoev (coordinator of IRSES project) on the topic: "New challenges related to animal health aspects of mycotoxins and by Prof. Mike Dutton (representative of the partner UJ – South Africa) on the topic "New challenges related to human health aspects of mycotoxins.
• 4) Participating in 42nd National Convention of the South African Chemical Institute (SACI) on 29th November up to 4th December 2015 in Durban, South Africa and Poster presentation by Siwe Noundou on the topic: "Erythrina caffra: A broad spectrum of biological activities".
• 5) Participating in National Conference of Young Researchers "Biological Science for better future" on 30-31 October, 2015 in University of Plovdiv, and delivering a presentations by Prerna Agarwal, Yanka Karamalakova, Manish Adhikari on the topic: "Aqueous root extract of Glycyrrhiza Glabra: An comparative study of the reaction with DPPH".
• 6) Participating in National Conference of Young Researchers "Biological Science for better future" on 30-31 October, 2015 in University of Plovdiv, and delivering a presentations by Prerna Agarwal, Yanka Karamalakova, Manish Adhikari on the topic: "Glycyrrhiza Glabra: "Real Time" oxidative status of animals"
• 7) Participating in National Conference of Young Researchers "Biological Science for better future" on 30-31 October, 2015 in University of Plovdiv, and delivering a presentations by Prerna Agarwal and Yanka Karamalakova on the topic: "Investigation The Influence Of Natural Antioxidants On "Real Time" Oxidative Status Of Animals"
• 8) Participating in National Conference of Young Researchers "Biological Science for better future" on 30-31 October, 2015 in University of Plovdiv, and delivering a presentations by Manish Adhikari on the topic: "Y-radiation induced DNA damage attenuation by Nano-silymarin:"
• 9) 20.01.2015 – Meeting between Project coordinator Prof Stoev and the DRDO visitors Prerna Agarwal and Manish Adhikari with Prof. Gadjeva and her team in Dept. of Chemistry in Fac. of Medicine in Trakia University (Bulgaria) in regard to some future studies and students qualifications to be undertaken as EPR (Electron Paramagnetic Resonance), NIRS (Near Infrared Reflectance Spectroscopy), etc., under the IRSES project.
• 10) 10.10.2015 – Meeting between Project coordinator Prof Stoev and the RU visitor Hilary

Ihesinaulo Ezuruike with Prof. Gadjeva and her team in Dept. of Chemistry in Fac. of Medicine in Trakia University (Bulgaria) in regard to some future studies and students qualifications to be undertaken as EPR (Electron Paramagnetic Resonance), NIRS (Near Infrared Reflectance Spectroscopy), etc., under the IRSES project.
• 11) Participating in National Seminar on “Challenges of Climate Change and Green Environmental Solutions” on 10th December, 2016, organized by Chaudhary Charan Singh University, Meerut, India. Via presenting the paper “Impact of climate change on mycotoxins in food: management interventions by herbs of Indian, European & South African origin" by Dr Rajesh Arora from DRDO.
• 12) Invited lecture of prof. Ivan Dinev under the WP7 as part of a research collaboration programme on mitigation of mycotoxins on the topic “Review on the incidences of some major pathologies of leg skeleton in broiler chickens and broiler breeders related to poor animal welfare” in Department of Animal Health at the Mafikeng Campus of the North-West University (NWU)
• 13) Invited visits and meetings of Prof Ivanov and prof Stefanov in Rhodes University with Prof William Froneman (Zoology), Prof Alan Hutchinson (Electron Microscopy Unit), Prof Martin Hill (Entomology), Prof Adrian Craig (Zoology), Prof Susanne Vetter (Botany), Prof Rosmary Dorrington (Microbiology), Dr Caroline Knox (Microbiology), Prof Joanne Dames (Biochemistry), Prof Brett Pleschke (Biochemistry), Dr Leonie Goosen (Pharmacy), and Dr Fanchesca Porri (South African Institute for Aquatic Biology – SAIAB) in regard to the advance of the IRSES project and future collaborations on some particular topics (November-December, 2016).
• 14) Invited discussions of Prof Ivanov and prof Stefanov with researchers in the Department of Chemistry, incl. with Dr Xavier Siwe Noundou (Natural Products Chemistry), Prof Tebello Nyokong (Nanotechnology Innovation Centre), Dr Vincent Smith (Crystallography), and Dr David Khanye (Inorganic and Medicinal Chemistry) on the achieved results under the IRSES project and elaboration of mixtures herbal extracts, having a protective effect against toxicity of target mycotoxins or improving wound granulation and immune response of animals as well as in regard to elaboration of target publications (1st week of December).

• DRAWBACKS AND PROBLEMS WE FACED

Overview of the activities carried out by the partnership; Identification of problems encountered and corrective action taken:

ETHICAL ISSUES

All chicks/mice were fed ad libitum on standard complete commercial feed suitable for their species and their age. Fresh drinking water was available ad libitum. The chickens were housed in wire-floor cages with continuous infrared lighting at a temperature and ventilation suitable for their age (ensuring the respective minimum area per bird, minimum enclosure size and height or length of feed trough per bird as given in Table 8.1 of Directive 2010/63/EU).

The mice/rats were housed in standard cages with 12 hour light cycle and floor area (above 450 cm2) and minimum enclosure size (above 800 cm2) or height (above 18 cm) per animal suitable for their body weight according to the rule of the respective Institutional Animal Ethics Committee and the requirements given in Table 1.2 of adopted Directive 2010/63/EU.

The ongoing monitoring of animal-welfare was ensured by veterinary care, which was available at all times and staff members were responsible for the care and welfare of animals. At the end of the experiments on the protective effects of various herbal additives, the respective animals were slaughtered at the respective slaughterhouses according to the rule accepted in each slaughterhouse (electrical stunning for chickens, etc.) or via using CO2 and euthanasia for rats/mice in compliance with the table 3 of methods for animal killing of adopted Directive 2010/63/EU.

Blood for clinical biochemistry and immunological investigations was taken after respective premedication and narcosis from the wing vein (in chicks), whereas tissues for pathomorphological

investigations was taken at slaughter time in the respective slaughterhouses or after the euthanasia of rats.

The incisional wounds in animals (pigs/rats/cattle, etc) created during the regular surgical interventions (such as castrations or other routine operations in the Department of Surgery) after the respective regular narcosis (premedication) and the local anesthesia as well as after the respective regular treatments were additionally treated by newly created herbal extracts/(unguents or sprays) for studying their stimulating effects on granulation tissue.

All “in vivo” or “in vitro” experiments were funded under the given sources of funding and therefore all Ethical issues required by the respective funding bodies and countries were already answered and the experiments already performed. The respective Animal Care Ethic Committees in each country has approved the study protocol and the rabbits/chicks/mice/rats in the all experiments were housed, maintained and slaughtered in accordance to the relevant international rules and recommendations (including adopted Directive 2010/63/EU from 22 of September which updates and replaces the 1986 Directive 86/609/EEC on the protection of animals used for scientific purposes to firmly anchor the principle of the Three Rs, to Replace, Reduce and Refine the use of animals) as well as to the Welfare Regulations in each country, where the experiments will be performed. The rabbits experiment in Hungary has been also authorised by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office. The 3 Rs principle (Replacement, Reduction and Refinment) was rigorously applied in each country and “in vitro” studies such as MTT assay or Comet assay (using Cac-40 intestinal cell line or lymphocytes) was performed where it is possible to evaluate the role of plant extracts in protecting the cytotoxic effects of OTA or FB1 or DON and to avoid the “in vivo” studies in which the minimum number of respective animals was also used. No animals were exposed to any pain or sufferл (all ethic authorizations for animal experiments are enclosed as appropriate from TU-Bulgaria and UNIKAPOS-Hungary).

DRAWBACKS AND PROBLEMS WE FACED

-Most of my colleagues, participated in our IRSES project have possibilities to realize only 1 month or shorter visits to our partners or beneficiaries – unfortunately IRSES project is not suitable for short time visits and the money available for 1 month can only cover the ticket price. Therefore, my proposal is to suggest some amendments in the financial rules of IRSES projects – such as some additional money for ticket to be ensured for short-time visits.

-Another problem we faced is the customs taxes and duties which we had to pay for the herbs received from South Africa for our experiments in Bulgaria and Hungary (more than 400 Euro were paid by our Hungarian colleagues for customs duties and taxes, etc).

-We also had a PROBLEM with the Regional Service of Phytosanitary Control and the Customs Service in Sofia, where our imported herbs from S. Africa via DHL were retained (consignment 6078281145) and we were asked to make a registration and to pay some taxes as importer of herbs, etc.

-There was a problem with realizing the secondments of Bulgarian researchers to India and part of them had to be postponed, because of some health problems of the seconded researchers to DRDO-India. This happened because the food in India is very spiced and hot and all of our visitors from Bulgaria had some stomach or gut health problems during their visits - they are not used to eat such hot food as the local people there. This problem is already partially resolved via some agreements with the owner of the private Persona Hotel International in New Delhi (the food which is usually prepared in this hotel is not extremely hot and spiced).

-ALL MENTIONED ABOVE PROBLEMS WERE SUCCESSFULLY RESOLVED.

8. ADDITIONAL INFORMATION

Additional information, which may be considered useful to assess the work done during the reporting period:

Attachments	40-breefing-IRSES project-2015.pdf, 38-Book-Food security-proof-2017.pdf, 37-E-book-Lambert-2015.pdf, 30-ETPh-review.pdf, 31-ChemBiolInt-BEN-enigma-review.pdf, 19-20-CompetitionScienceYouth-Plovdiv-2016.pdf, 18-IntCongressMedSci-Sofia-2016.pdf, 17-IntSciConf-Stara Zagora -2016.pdf, 16-AnnualSciConferenceMilitaryUniv-V.Turnovo-2016.pdf, 15-NatSeminarClimateChangesGreenEnvSolution-India-2016.pdf, 14-IntCongressMedSci-Sofia-2015.pdf, 13-NatConfBiolSciBetterFuture-Plovdiv-Agarwal-2-2015.jpg, 12-NatConfBiolSciBetterFuture-Plovdiv-Agarwal-2015.pdf, 11-UJ-EthnoPharmacology-Zingue-2016.pdf, 10-UJ-BMC ComplementaryAlternMed Zingue-2016.pdf, 9-UJ-EthnoPharmacology-Noundou-2016.pdf, 8-UJ-EthnoPharmacology-Fodjo-2017.pdf, 7-Paper-chick-herbs-India-in press.pdf, 6-Paper-chick-herbs-SA-in press.pdf, 5-Paper5-Proceeding-Karamalakova-2016.pdf, 4-Paper4-SciTechMedBiol-Karamalakova-2016.pdf, 2-Paper2-JBiolSciBiotechnol-Agarwal- 2015.pdf, 1-Paper1-RegToxPharm-G.Nikolova -2016.pdf, 3-Paper3-Agarwal-2016.pdf

Date:

Person in charge of the project for the beneficiary(ies):