SAFETY EVALUATION OF *AGARICUS SUBRUFESCENS* VARIETIES AND THEIR PRODUCTS OF THERARPEUTIC INTEREST OR FOR DISEASE PREVENTION

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ABSTRACT

Mushrooms are food traditionally consumed in Europe, Asia and America. They are being studied for medicinal benefits. Extensive studies have shown that *Agaricus subrufescens* (*A. blazei* Murrill or *A. brasiliensis*) has anticancer properties. A comparative study of *Agaricus subrufescens* strains (from Brazil and France) is presented herein using *Agaricus bisporus* (champignon de Paris) as control. *In vivo* OCDE test were performed to evaluate either tolerance and/or acute and sub chronic toxicity in rats and mice.

Our data reveal that all *A. subrufescens* strains are not toxic, either *in vivo* or *in vitro*, except some locomotor hypoactivity. All show a preventing effect against carcinogenesis, including *A. bisporus.* This is the first time that this mushroom is shown to be effective, even thought it is clearly less effective than *A. subrufescens*. However no anti tumour effect is found using Balb-c mice implanted with leukaemia cells. Furthermore they elicit slight cell growth stimulation at the concentrations tested in vitro, in Hep G2 (human hepatoma cells) and Neuro 2a (mouse neuroblastoma cells). The most active is *A. subrufescens* from Brazil.

These mushrooms do have many bioactive compounds, different from the polysaccharides that need to be isolated and characterised for their curative properties following accurate evaluation of toxicological effects. Indeed there is clearly a lake of information on toxicological assessment (acute and chronic toxicity) of compound such as agaritine, blazein among others and of the whole mushroom *A. subrufescens* itself, and overall on epidemiological data linking the consumption of *Agaricus* sp and eventual prevention and/or pathologies.

Keywords: *Agaricus subrufescens*, *Agaricus bisporus*, Safety, Prevention, Rodents

INTRODUCTION

Agaricus subrufescens Peck is also known as *Agaricus blazei* Murrill and/or *Agaricus brasiliensis* Wasser [1, 2] and has other common names, such as "piedade mushroom" or "Cogumelo do sol" in Brazil, "Himematsutake" in Japan and "Almond mushroom" in United States of America. It is named as ABM in several articles. This *Agaricus* is rich in substances that are expected to modulate biological functions in mammals. Various active compounds that can potentially be used to treat or prevent different diseases have been reported [3-5]. This mushroom has been used as a medicinal food for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis and is known to impact putatively the immune system [4]. Potential effective compounds of this mushroom can be isolated either from their fruiting bodies, or even from pure culture of mycelia and culture broth filtrate.

Consumption of cultivated mushrooms or their extracts is suggested to have several benefits. Paradoxically no real safety study on wild *A. subrefescens* or *A. bisporus* has been performed. ABM is currently studied and commercialised as a potential functional food or beverage (tea). Several studies on many substances of this mushroom such as polysaccharides, sterol, sodium pyroglutamate, lectins and RNA-protein complex have been reported as potential bioactive substances [1-4]. Among those molecules the most extensively studied are the polysaccharides for their anti tumour effect. One has to admit that polysaccharide structures from isolated fractions are poorly resolved and not forceful. These enriched fractions of polysaccharide could be contaminated by undetected compounds (possibly strongly cytotoxic ones). Many questions arise that fully justify the proposed studies aiming to evaluate the safe use of this mushroom and its eventual pure substances of interest.

What do we learn from the literature on *A. subrufescens* Peck, *A. blazei* Murrill and/or *A. brasiliensis* Wasser?

Table 1: Bioactive compounds from *Agaricus subrufescens*

Bioactive Compounds	Main Potential Effects	References
Polysaccharides		[3], [6], [7], [8]
β-(1,6)-glucan	Immuno-active involved in	[6]
α-(1,4)-; β-(1,6)-glucan	anti tumour effect and/or	[9], [10]
α-(1,6)-; α-(1,4)-glucan	prevention; antimicrobial	[11]
β-(1,6)-; β-(1,3)-glucan		[11]
β-(1,6)-; α-(1,3)-glucan		[11]
Glucomannan		[12]
β-(1,2)- β-(1,3)-glucomannan		[13], [14], [15]
Glucan-Protein complex	Oncogenesis prevention	[6], [9], [10]
Lectin	Anti-tumour	[17]
Riboglucan	Anti-tumour	[18]
Ergosterol	Anti-tumour	[4]
Sodium pyroglutamate	Anti-tumour	[19]
RNA-protein complex	Anti-tumour	[20]
Agaritine	Anti-tumour	[21]
Blazein	Anti-tumour	[23]

The average composition of mushrooms is normally 90% water, 2-40% protein, 2-8% fat, 1-55% carbohydrate, 3-32% fibber, yielding 8-10% ash [5]. Several categories of molecules are supposed to be involved in beneficial effects and most of the molecules categories found in *A. subrufescens (A. blazei* Murill) are common to the entire fungal kingdom. Active compounds found in *A. blazei* are listed in Table 1. The active compounds isolated from *A. subrufescens* are believed to be mainly polysaccharides [3], [6], [7], [8] β-glucan [9], [10], [11], [14] and glucomannan [12], [13], [14] or riboglucan [18] (Table 1). Additionally, in *A. blazei*, a protein (or glycoprotein), Glucan protein complex, a lectin [17], [18], originally found associated to β (1, 6) -glucan, were characterized and claimed to be anti-tumour (Table 1). Recently, it has been shown that *A. blazei* sp do contain aromatic hydrazines, especially agaritine [21] (Table 1) and its derivatives [22]. More recently, blazein a steroid derivative found in *A. blazei (A. subrufescens)*, was shown to kill Human lung cancer LU99 cells but not normal Human lymphocyte [23]. Agaritine extracted from *A. blazei* was also showed to kill leukemic cell in vitro. Its cytotoxicity is triggered cell apoptosis with an IC50 in the range of 2.7 to 16 µg /ml

depending on the cell line [24], [25]. Agartine is found at similar concentration in both *A. blazei* and *A. bisporus* (approximately 1.8 mg/g dry weight) [21].

Several authors found antibacterial properties of glucan against *Staphylococcus aureus* [25] rendering this compound an attractive immunotherapeutic agent even against diverse virus [27], [28], [29]. [30].

Further, these immune modulators properties were also described for several diverse mushrooms polysaccharides extracts [29]. This potential effect was also clearly reported for *Agaricus blazei* extracts by several authors such as for example Mizuno *et al.* [31]. Therefore it is believed that mushrooms glucans or protein-polysaccharides complex have host mediated immune-modulator properties that trigger anti-cancer and anti-microbial effects. Furthermore, the water-soluble extracts (proteo-glucans) and AndoSan from crude *A. blazei* up regulate the in vitro maturation of dentritic cells [32], [33]. Ex vivo experiment on total heparinised blood of volunteers and in vitro experiment show that the monocytes-derived dendritic cells from peripheral blood mononuclear cells produce an increase level of cytokine and chemokines. The most abundant cytokines after *A. blazei* Murill stimulation were mainly pro-inflammatory cytokine and chemocytokines IL-8, G-CSF, TNF-α, IL-1β, IL-6, IL 17 and MIP-1β. The synthesis of IL-2 and IFN γ is also reported but not of IL-12 as previously said. Therefore only some Th1-type and Th17 type cytokines were up regulated whereas no anti-inflammatory or Th2 cytokines were stimulated in vitro. Whereas ex vivo there is a release of different cytokines including Th-1, TH-2, pro inflammatory or anti-inflammatory as well as the chemokines and leukocyte growth factor. Therefore this *A. blazei* immunostimulatory extract proceed mainly by pro inflammatory chemocytokine and cytokines in vitro or ex vivo.

The inhibition of pro-inflammatory cytokines appears to be dose dependant [34]. This is in contradiction with most of the tests made in murine models with different *A. blazei* extracts. This contradiction can be explained only by the capacity of certain glucans to cross the murine intestinal barriers. Ex vivo experiment with healthy volunteers heparinised blood controversially showed stimulation of all pro-inflammatory cytokines tested as expected. More recently, a clinical test with patients suffering of inflammatory bowel disease (IBD) inflammatory disease of colon and intestine has been conducted [33]. Patients with Chron's disease (CD) widely regarded as an autoimmune disease, and ulcerative colitis (UC) were exposed orally to *A. blazei* enriched mixture (AndoSan). After 12 days ingestion, the *A blazei* extract promotes in these patients anti-inflammatory effects with no side effect.

There are opposite effects between *in vivo* and *ex vivo* assays with normal patients, pro-inflammatory cytokines and chemokines are controversially down regulated and up regulated respectively. This discrepancy are tentatively explained by the antioxidant potential of *A. blazei* [35], [36] and the limited absorption of large macromolecules complex or bioactive molecule such β-glucans across the intestinal mucosa to the reticulo-endothelial system and blood through the gastrointestinal tract. With CD and UC patients the same conclusive explanation is proposed, but the intestinal barrier is hypothesized to be presumably selectively permeable to certain β-glucans bioactive fragments to body fluids (blood and lymphoid system) as in murine [33]. This is yet too speculative. Does the presence of molecules that differ from polysaccharides or PAMPS (Pathogen Associated Molecular Patterns) exert immunosuppressive effect on the innate immune system?

It appears clearly that most of the studies were performed on mixtures of substances (even through purified) allowing these contradictory data in which the same substance does something and the contrary. Thus purification and specific studies are absolutely needed in each of the structural groups of substance.

All together it appears that *A. blazei* extracts do exert both immunostimulatory and immunosuppressive effects. Globally glucan, proteoglucan complex peptide-glucan appeared to be rather immunostimulating and promoting indirectly anti cancer benefits. Whether

immunosuppression is triggered by the same categories of molecules such as more or less branched polysaccharides or proteoglucans remains unclear and too speculative. However, because of the immunosuppressive potential, safety of chronic exposure to dietary edible commercial *A. blazei* should be evaluated.

The biological material to be studied consists of original Brazilian and European *A. subrufescens* cultivated on conventional compost using *A. bisporus* as reference material for comparison. The whole study has then been designed to investigate on mushrooms safety and new products of therapeutic interest or for disease prevention: fruitbody composition, nature of the metabolites produced by this species and their toxicological or pharmaceutical effects. Preliminary data are included herein.

MATERIALS AND METHODS

Production of mushrooms extracts. *A. subrufescens* CA454 (CGAB collection, INRA, France) formerly *A. blazei* (strain ATCC 76739) from Brazil and at the origin of commercial strains, *A. subrufescens* CA 487, European strain of French origin (CGAB collection, INRA, France) and *Agaricus bisporus* 30A (commercial strain, France Mycelium), were grown in the facilities of the research group MycSA, INRA France.

Fungal preparation for animals' treatment: A mass of 100 g of freeze-dried mushrooms was ground with a blender; powder was added to 100 mL of ultra-pure sterile water (Milli-Q plus, Millipore, France) at room temperature for 30 min with stirring to yield a suspension of mushroom (mother suspension) which was subsequently diluted to 0.5g and 0.25g/ml, used for gavages. When needed suspensions were centrifuged at 2000 g for 20 min to yield supernatants used for intra-peritoneal injections.

Fungal water extraction methods for in vitro alternative assays: Hot water hydrophilic extracts: A mass of 10 g of freeze-dried mushrooms was ground with a blender; powder was added to 100 mL of ultra-pure boiling water (100 °C) for 6 hours with stirring [37]. The suspension was then centrifuged at 2000*g* for 10 min at 20 °C. The supernatant was freeze dried and the resulting powder weighted. For *in vitro* alternative assay, a stock solution was prepared by re-suspending the powder directly in cell culture medium without foetal calf serum and dilution was made with the same medium.

Cold water hydrophilic extracts: A mass of 10 g freeze dried mushroom was ground and lyophilized. The matrix was put into a flask by adding 100 mL of sterile ultrapure water. The vial was placed 1 h at room temperature and then at 4 °C for 48 h with agitation. The suspension was then centrifuged at 2000xg for 10 min at 4 °C. The supernatant was recovered, lyophilized and the resulting powder weighted. For *in vitro* alternative assay, stock solution and diluted solutions were made as described above.

Animal testing. Male and female Swiss mice (22 ± 2 g) and male and female Wistar rats (200 ± 20g) were weighed and stall feeding one week before the test. They were divided into two groups consisting of four subgroups of five animals.

Group 1, "Evaluation of tolerance and safety": Groups of 10 males and females Wistar rats separately caged received each day for a week, 1 gram of mushroom suspension per kilogram of body weight. They were kept under observation for an additional 15 days after the end of treatment for any signs of toxicity or behavioural modification. They were weighed daily and at the end of the experiment, 21 days (n = 10). Similarly, mice have been treated to evaluate the tolerance.

Group 2, Evaluation of acute oral dose "(limit test)": Animals were divided into groups of 5 males and females separately caged and received mushroom suspension either 1 g / kg of body weight or 2 g / kg of body weight in a single intra gastric tube.
"Evaluation of acute oral dose (limit test)": The animals received by gavages a dose of either 2g/kg or 5g/kg body weight and kept under observation for two weeks. The OECD test 423 was conducted with female rats of the Sprague Dawley strain F344 (SPF Caw) of about 8 to 12 weeks at the beginning of the test. These tests were performed in GLP conditions at the Phycher-Biodevelopment Laboratory in Cestas (France).
In vivo Model to assess anti carcinogenesis effect: The rat gut carcinogenesis model of methylimidazol and phenylimidazol which are known to promote cancer has been used. F344 rats were treated with aromatic amine derivatives (methylimidazol and phenylimidazol) for one week. Then they received crude extracts of *Agaricus sp* or suspension of mushrooms powder, or extracts daily by oral route (0.25, 0.5 and 1g/kg body weight) for three months. Intestines from all animals were examined following staining with methylene blue (1% in NaCl 0.9%) to assess the pre-neoplasic changes (different foci following histological observation under microscope; magnification 40).
Preliminary test in cancer model mice: Animals, Balb-c male and female used to develop the mouse model of cancer were treated (n = 5) orally and / or by the intra peritoneal route (supernatant of mushroom suspension following centrifugation at 2000g for 20min) with a dose of extract corresponding to 0.5, 1 and 2 g of fresh mushrooms each day for 15 days. They were observed daily before treatment and kept under observation for 15 days after the end of treatment. Then 4 groups of 10 mice were treated following transplant of L1210 and/or SP2-O leukaemia cells in the peritoneal cavity. After 10 days of incubation, animals were treated daily with either pure sterile saline (NaCl, 0.9%) or extracts of *A. subrufescens* CA454, *A. subrufescens* CA487, and/or *A. bisporus* 30A. They were kept under observation until death.

In vitro assays. Cell culture: The HepG2 human hepatocarcinoma cell line (ATCC HB8065) was grown in MEM-Glutamax containing 1 g/l glucose supplemented with 10% foetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin and 1% non-essential amino acids. The Neuro2a mouse neuroblastoma cell line was cultured in RPMI 1640 containing 2g/l glucose, 2% L-glutamine and supplemented with 10% foetal calf serum, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin. All cells were routinely grown in 75cm^2 and incubated in humidified atmosphere consisting of 5% CO2, 95% air mixture at 37° C.
Cytotoxicity assay by MTT test: Before treatment, cells (10^5 cells/ml) were cultured (100 µl) in 96-well plates for 24 h. The medium was removed from the wells and cells were exposed (triplicates) during 48 h to different concentrations of fungal extracts amended into the medium without foetal calf serum (100µl/well). Plate design omitted marginal rows and columns and 6 wells were used for cell viability control. The cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After treatment, the medium was replaced by a FCS-free medium containing 0.5 mg/ml MTT (Sigma) for 2 h at 37°C. The medium was discarded prior the addition of DMSO to dissolve the formazan. The absorbance was read at 495 nm with a microplate reader (LT4000 Labtech, France). Viability was expressed as the percentage of mean absorbance of the cell viability in control (100% viability).

Statistical analysis. Data were expressed as mean ± SEM or absolute value where needed and always compared to controls by using Analysis of Variance and Tukey Honestly Significant Difference tests. Statistical analysis was done by using the R software (v2.3.1, 2006) and *P* values less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

First we found during the grinding and preparation of diverse solutions of the mushrooms that the strains of *A. subrufescens* showed different colours: CA487 yielded a yellowish white suspension in cold extracts while those of CA 454 were yellow curry. Comparatively suspensions of *A. bisporus* 30A were light brown. The colour differences between the suspension of strains of *A. subrufescens* (CA 454 and CA 487) and *A. bisporus* A30 might be related to a difference in composition including that of the active substances.

Evaluation of Tolerance and Acute Oral Dose "(Limit Test)". Animals, rats and mice (group 1) showed no toxic effect during treatment or within the 15 days of surveillance following treatment, excepted, those treated with the suspension of powder of *A. subrufescens* strains at a dose of 2 g / kg. In these animals a decrease in locomotor activity and a soothing effect were observed. The gains in body weight did not change significantly compared with controls who received only water. The relative weights of major organs (liver, kidney, heart, brain, genitals) have not changed either.

Group 2 animals (rats and mice) showed no acute toxic effects with the exception of animals receiving 2 g / kg body weight of *A. subrufescens* CA454. Similarly, decrease in locomotor activity and a soothing effect were observed within 24 to 72 hours. As before there were no other changes.

The OECD 423 test conducted under the conditions of COFRAC GLP accreditation revealed in the rat a LD50> 5g/kg of body weight.

Treatment resulted in no significant effect either by the intra peritoneal route (supernatant of suspension) or by the oral route. The product is well tolerated and does not lead to toxic effect except locomotor hypoactivity with all the suspensions *of A. subrufescens* CA454 and CA487. Neither weight gain nor the relative mass of the organs were affected. Safety testing (12 weeks) and oral acute toxicity tests show a difference between CA454 and CA487. The first one involved an unexpected and currently unexplained locomotor hypoactivity. We were unable to connect this hypoactivity (during the first 2-3days) with the swelling of foot pads as described by Chan *et al.* [38] in connection with a pro-inflammatory (TNF-alpha) and hypersensitivity phenomena. Altogether these data suggest that the extraction protocol using water (room temperature, and centrifugation) is not sufficient to extract all substances of the mushrooms. Hence extraction following boiling is subsequently proposed. Indeed, Chan *et al*. [38] have performed their extract preparation in hot water (80-85°C) and finished by sterilization at 105°C for 10 min. They have certainly extracted additional substances as compared to our extracts. The confirmation of this hypothesis is underway with further solid state NMR studies of our recent extracts at high temperature.

The dose of 5 g of fresh mushrooms per kilogram body weight would make 300 g of fresh mushrooms for a person of 60 kg, without acute toxic effects. However, one cannot exclude chronic or sub chronic effects, following daily or frequent ingestion of such a dose of mushrooms or the corresponding extract, for several weeks or months.

Such a chronic toxic effect (tumour-promoting activities) has been revealed in rodents fed with feeds containing *A. blazei* Murill in Japan [23]. This finding led to a severe restriction of commercialisation of *A. blazei* Murill, whatever the form, for human consumption by the Ministry of Health, Labour and Welfare of Japan in 2006.

This apparent lack of toxicity does not exclude the presence of active substances. Indeed many situations can be considered. It can there be a substance having an effect on immunity as described in the literature, immuno stimulant [38] or immunosuppressant [33,34].

In Vivo Model to Assess Anti Carcinogenesis Effect and Preliminary Testing of Anti Cancer Properties. The administration of the *Agaricus* powder suspension or fungal extracts (0.25, 0.5 and 1g/kg Body Weight) elicits positive effect on intestinal carcinogenesis. All mushrooms were effective in preventing gut-induced carcinogenesis (Tables 2-4). The relative preventions were respectively of 72%, 64% and 56% for CA454, CA487 and A30 (Table 5). For the later this preventive effect is unexpected and indeed demonstrated for the first time. This justifies the use of *Agaricus sp* in cancer prevention by millions of people all over the world.

Table 2: Number of histological foci observed in intestinal tissues of rats treated by *A. subrufescens* suspension and/or abstracts (0.25g/kg BW) following induction of carcinogenesis process with phenylimidazolic compounds. Each number represents the sum of foci counted in 4 different fields by slice.

Treatments	**Pre neoplastic lesions observed by slices of intestinal tissue**			
	Type 1 lesions (Big size foci)	**Type 2 lesions (Foci with thick margin)**	**Type 3 lesions (Extended Foci)**	**Type 4 lesions (Foci with peripheral zones)**
Untreated Controls (n=5)	1	0	0	0
A. subrufescens CA 487 (n=5)	3	3	2	3
A. subrufescens CA 454 (n=5)	1	2	2	4
A. bisporus A30 (n=5)	4	4	2	4
Imidazol-Treated Controls (n=5)	6	9	5	8

Table 3: Number of histological foci observed in intestinal tissues of rats treated by *A. subrufescens* suspension and/or abstracts (0.5g/kg BW) following induction of carcinogenesis process with phenylimidazolic compounds. Each number represents the sum of foci counted in 4 different fields by slice.

Treatments	**Pre neoplastic lesions observed by slices of intestinal tissue**			
	Type 1 lesions (Big size foci)	**Type 2 lesions (Foci with thick margin)**	**Type 3 lesions (Extended Foci)**	**Type 4 lesions (Foci with peripheral zones)**
Untreated Controls (n=5)	1	0	0	1
A. subrufescens CA 487 (n=5)	2	3	1	5
A. subrufescens CA 454 (n=5)	2	2	1	3
A. bisporus A30 (n=5)	3	5	1	3
Imidazol-Treated Controls (n=5)	4	6	4	10

Table 4: Number of histological foci observed in intestinal tissues of rats treated by *A. subrufescens* suspension and/or abstracts (1g/kg BW) following induction of carcinogenesis process with phenylimidazolic compounds. Each number represents the sum of foci counted in 4 different fields by slice.

Treatments	Pre neoplastic lesions observed by slices of intestinal tissue			
	Type 1 lesions (Big size foci)	**Type 2 lesions (Foci with thick margin)**	**Type 3 lesions (Extended Foci)**	**Type 4 lesions (Foci with peripheral zones)**
Untreated Controls (n=5)	0	1	0	1
A. subrufescens CA 487 (n=5)	4	3	2	2
A. subrufescens CA 454 (n=5)	3	2	3	2
A. bisporus A30 (n=5)	4	5	3	1
Imidazol-Treated Controls (n=5)	8	10	6	7

Table 5: Sum of all types of foci for each mushroom and percent of prevention following normalisation of values, taking into account that 5 foci/group of 5 animals is not significant.

Treatment	Total pre-neoplasic foci	% of prevention
Untreated Controls (n=15)	5	100
A. subrufescens CA 487 (n=15) (0.25-1g/kg BW)	33	64
A. subrufescens CA 454 (n=15) (0.25-1g/kg BW)	27	72
A. bisporus A30 (n=15) (0.25-1g/kg BW)	39	56
Imidazol-Trémate Controls (n=15)	83	0

The doses used in our experiments were fixed at respectively 1/20 to 1/5 of the maximum dose experimentally ingested since LD50≥ 5g/kg BW (as obtained in a previous work, BRG national project in 2007) that is 0.25, 0.5 and 1 g/kg BW. From the lower dose of 0.25 g/kg BW to the rat, we already observed almost the maximum protective effect. It appears that there is no dose-effect relationship in this protective effect. Interestingly Johnson *et al.* [33] and Forland *et al.* [34] have given to human a dose of 60 ml per day of a solution containing 340g/L that makes 0.34 g/kg BW for a person of 60 kg. These doses are quite similar to those given to the rats in the present experiments, however in our case and having the end-point of pre neoplasic changes, we found a very significant protective effect in the rats whereas in [33] and [34] immunosuppressant effects in humans were observed. If the preventive effect observed is triggered by immune stimulation as forecasted or hypothesised by several authors [25, 38], our data appear incongruent with those of Johnson *et al.* and Forland *et al.* [33, 34]. But, if the tumour preventing effects are triggered by cytotoxicity against modified cells bearing rapid growth rate [39, 40], then our data are congruent with the above studies [33, 34], since the immunosuppressant effect observed could be triggered by cytotoxicity.

Based on the present data our preliminary conclusion is that *A. subrufescens* CA454, CA 487 and *A. bisporus* 30A might have tumour preventing properties. Then further step was investigation on anti tumour properties. This has been done using the mouse transplanted-tumour model. Balb/c mice have been treated with similar doses of mushroom extracts by both intra

peritoneal and oral routes after having been injected with L1210-SP2O leukaemia cells. We were unable to prevent death of animals. Thus no anti-tumour effect can be claimed for *A. subrufescens* CA 454, CA 487 and *A. bisporus* A30, at least with these extracts.

In Vitro Assays. After obtaining cold and hot hydrophilic extracts they were tested in vitro on two cell lines Hep-G2 and Neuro-2A. The concentrations used in vitro were chosen from our in vivo data, considering that we should not put more than 1000 µg/ml when 1 g/kg BW is effective in vivo. Further we have seen that most of the research groups never exceeded 600 µg/ml of extracts or polysaccharides in their culture medium. Our *in vitro* data revealed no effect until concentrations of 200 µg/ml, then slight mitogenic effect, when cell growth was stimulated somehow between 200 and 500 µg/ml and then no clear inhibitory effect was observed above these concentrations (Fig. 1, a,b,c and d). Hep-G2 cells were more sensitive to these extract than Neuro-2a cells. Since Hep-G2 cells originate from the liver, it could be that liver cells are more sensitive to *A. subrufescens* than other cells. Indeed several authors found some liver damages (fulminant hepatitis) with extract of *A. subrufescens* sp [5, 41], *A. subrufescens* strains were more active than *A. bisporus*, with CA454 being the more active.

These data are congruent with the stimulatory effects observed on immune cells both *in vitro* and *in vivo*. Purification and characterization of active substances could help finding in the future any curative effect borne by these mushrooms. At present our *in vitro* data show some growth stimulatory effects at the tested concentrations. It is noticeable that the cold extracts are more effective than the hot extracts (Fig. 1 a, b, c and d). This observation could be due to the partial destruction of some active mitogenic substances by heating or differential extraction.

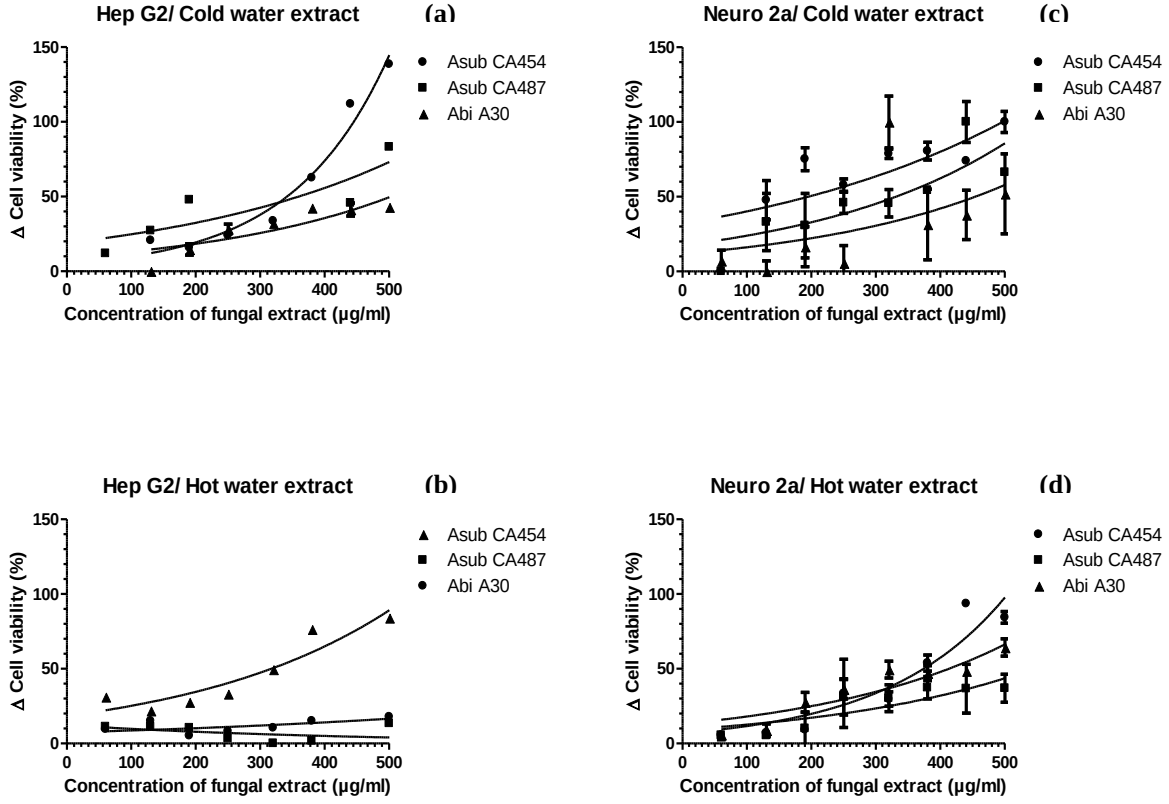

Figure 1: Effects of hydrophilic extracts of *A. subrufescens* sp (Asub) and *A bisporus* (Abi) on the viability of Hep G2 and Neuro-2a cells; (a) and (c) = cold water extracts of total mushrooms; (b) and (d) = hot water extracts of total mushrooms.

CONCLUSION

The final goal of the toxicological and chemical studies is to give safety information and to promote any of these mushroom strains which will prove to be of interest either as food or as drug to be used for curative or preventive purposes so that implementation of its culture at a large scale will proceed of sustainable and profitable agriculture.

Agaricus subrufescens or *A. blazei, A. brasiliensis* has been consumed by human for decades. Due to the popularity, it is increasingly cultivated in many countries for its putative medicinal properties. Many scientific studies reported various medicinal benefits i.e. anti-cancer, anti-microbial anti oxidant and anti inflammatory activities. Although these mushrooms have many bioactive compounds that could be developed for curative properties toxicological problems are still discussed. There is clearly a lake of information on toxicological assessment (acute and chronic toxicity) of compounds such as agaritine, blazein and of the whole mushroom *A. subrufescens* itself, and overall on epidemiological data linking the consumption of *Agaricus* sp and eventual prevention and/or pathologies.

Our data reveal that *A. subrufescens* strains of different origins are not toxic either *in vivo* or *in vitro* except some locomotor hypoactivity. Additionally they elicit slight cell growth stimulation at the concentrations tested *in vitro*. The most active is *A. subrufescens* (*A. blazei* Murrill) from Brazil whereas the most productive is the European species [42].

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