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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

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## Full Length Research Paper

## Effects of extracts from *Linum usitatissimum* on cell vitality, proliferation and cytotoxicity in human breast cancer cell lines

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The seeds of flax (binomial name: *Linum usitatissimum* L.) are well known for their high content of phytoestrogens. In the present study, extracts from roots, leaves and stems of flax were analysed for their content of compounds, which might have phytoestrogen-like properties, by pyrolysis field ionisation mass spectrometry. All extracts were tested on the human breast cancer cell lines MCF7 (estrogen receptor positive) and BT20 (estrogen receptor negative). Specific tests were applied for cell vitality ((3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (MTT) test), proliferation (BrdU test) and cytotoxicity (lactate dehydrogenase (LDH) test). In the flax root extract, the amounts of monolignols and polyphenols were three times higher than in the stem and leaf extracts. Even at higher concentrations, the root extract also was the least cytotoxic one of all extracts in MCF7 cells, while it showed a dose-dependent and much higher cytotoxicity in BT20 cells. Furthermore, at higher concentrations (> 100 µg/ml), the root extract reduced cell vitality in MCF7 significantly less than in BT20 cells and inhibited proliferation in MCF7 by up to 85%. Since flax root extracts induce significant inhibition of cell vitality and proliferation without performing strong cytotoxicity in the human mamma carcinoma cell lines MCF7, the potential phytoestrogens in flax root extracts could have beneficial effects in hormone-dependent tumours.

**Key words:** Flax, *Linum usitatissimum*, phytoestrogens, MCF7, cell proliferation, breast cancer.

### INTRODUCTION

Plants produce more than 100,000 different low-molecular-mass compounds, known as secondary metabolites. Amongst the most numerous are lignans, isoflavonoids and coumarins (Dixon, 2001). Being able to bind to the human estrogen receptors alpha and beta

(ER $\alpha$ , ER $\beta$ ), with higher affinity to ER $\beta$ , they were named phytoestrogens (Kuiper et al., 1997). In several publications, anti-proliferative effects of these substances on tumour (cell) growth were described (Lamartiniere et al., 1995; Zhou et al., 1999). They inhibit enzymatic activities

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involved in intracellular signal transduction, which are involved in the stimulation of cellular growth factors (Scholar and Toews, 1994). Phytoestrogens act also on the cellular metabolism by ER-independent mechanisms such as induction of apoptosis, inhibition of topoisomerase II and angiogenesis, or by anti-oxidative effects (Hostanska et al., 2004; Kulling and Watzl, 2003; Peterson and Barnes, 1991).

For several crude extracts, for example from peas, beans, pumpkin seed, red clover or soy beans, which contain phytoestrogens binding to ERs, an anti-tumour activity was demonstrated (Booth et al., 2006; Boue et al., 2003; Liu et al., 2001). A diet with flax seeds, which are very rich in lignans, significantly reduced MCF7 cell tumour size in mice (Adlercreutz and Mazur, 1997; Horn-Ross et al., 2000; Saarinen et al., 2006). Also, other parts of flax may be of interest, as it was shown that the cell vitality of the chorion carcinoma cell line Jeg3 was more strongly inhibited by flax root extracts than by flax leaf or stem extracts (Abarzua et al., 2007).

Flax is a food and fibre crop that is grown in cooler regions of the world, for example Canada, Russia and Germany. It is an annual plant, reaching a height of up to more than 200 cm. Anthesis is between July and August; the main growth phase is in May and June. The plant does not make great demands on soil and thus it can be easily cultivated.

The aim of the present study was the identification of putative phytoestrogens in methanolic extracts of leaves, stems and roots of flax and to test the effect of these extracts on cell vitality, proliferation and cytotoxicity in ER-positive and ER-negative human breast cancer cell lines *in vitro*.

## MATERIALS AND METHODS

### Preparation of extracts

Seeds of the flax *Linum usitatissimum* L., cultivar Barbara, provided by the Agricultural Research Institution (LUFA), Rostock, Germany, were sown on soil and grown under field conditions. The flowering plants were harvested, and leaves, stems and roots were frozen separately in liquid nitrogen and stored at -80°C until extraction. The extracts were prepared according to Luyengi et al. (1996) as modified by Matscheski et al. (2006). Plant material (20 g) was ground with liquid nitrogen in a mortar and extracted with 180 ml 100% methanol in a water bath for 15 min at 70°C by using a reflux condenser. The solution was cooled, filtered and evaporated almost to dryness. The extract was resuspended in 8 ml of distilled water and partitioned with ethyl acetate for five times. After drying, the ethyl acetate soluble fraction was dissolved in absolute ethanol to provide a stock solution of 100 mg/ml. The extracts were stored at -20°C.

### Molecular-chemical analysis of extracts

The extracts (stock solution) were screened by pyrolysis field ionisation mass spectrometry (Py-FIMS) (Schulten and Halket, 1986). It combines a temperature-resolved volatilisation during pyrolysis and a soft ionisation of molecules at high-vacuum (Beckey, 1977; Schulten, 1996). About 5 µl of the extract were

transferred to a quartz capsule that was placed in the micro-oven of a double-focusing Finnigan MAT 900 mass spectrometer (Finnigan, MAT, Bremen, Germany). The micro-oven heated the sample from 110 to 700°C in 12 min, and 91 magnetic scans were recorded for the mass range  $m/z$  15 to 900 (single spectra). The marker signal selection was based on the concepts of Schulten et al. (1989), Hempfling et al. (1991) and Hempfling and Schulten (1991). A detailed description of the Py-FIMS method and the statistical treatment of TII and data normalisation are provided by Sorge et al. (1993).

### Cell lines and cell culture

The human breast cancer cell lines MCF7 and BT20 were obtained from the Department of Human and Animal Cell Culture, Braunschweig, Germany. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich-Chemie, Germany), supplemented with 10% inactivated fetal calf serum (FCS, Biochrom, Germany), 1% antibiotics (penicillin/streptomycin, PAA, Germany) and 0.5% amphotericin B (PAA, Germany) under a humidified atmosphere (37°C and 5% CO<sub>2</sub>). MCF7 cell line is described to be ER alpha, beta and progesterone receptor positive. BT20 cell culture does not express the ER receptors (manufacturer's protocol). We confirmed this by immunocytochemistry.

### Cell vitality, cell proliferation and cytotoxicity

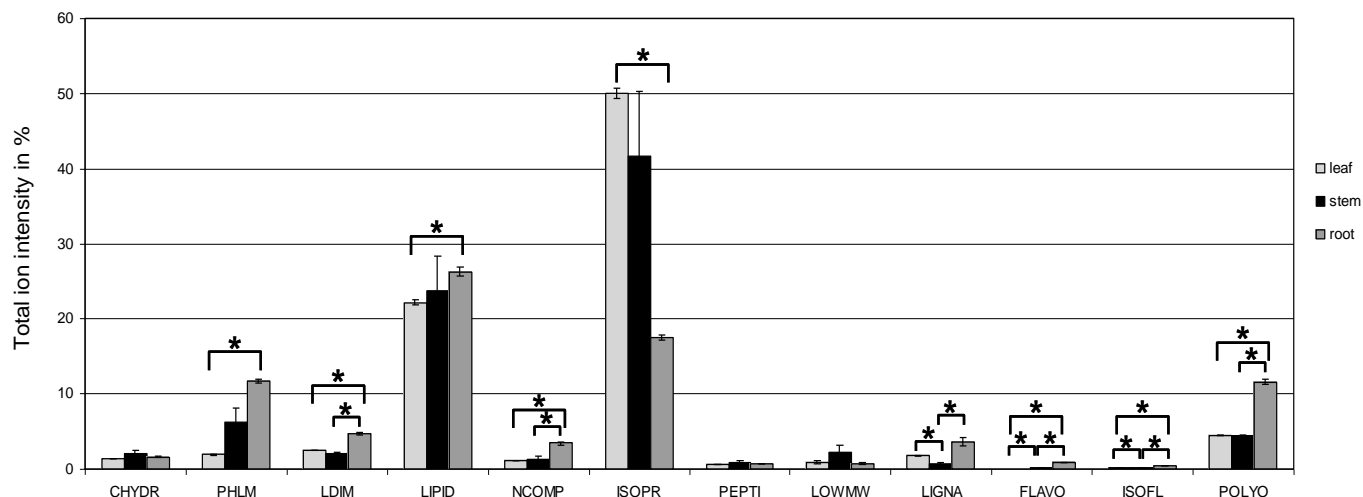
Cell vitality, cell proliferation and cytotoxicity in the human breast cancer cell lines MCF7 and BT20, treated with the extracts from *L. usitatissimum* were analysed by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method, a 5-bromo-2'-deoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (colorimetric), and a cytotoxicity detection kit (LDH kit for lactate dehydrogenase) as was recommended by the manufacturer (all Roche, Germany). For MTT, BrdU and LDH test,  $5 \times 10^5$  cells/ml were grown in 96-well tissue plates for 24 h at 37°C and 5% CO<sub>2</sub>. Subsequently, extracts and controls were added and incubated for 24 h at humidified atmosphere. Aliquots from the stock solutions of root, leaf and stem extracts were dissolved in absolute ethanol and added to the supplemented culture medium to give final concentrations of 0.01, 0.1, 1, 10, 50, 100, 500 and 1000 µg/ml (final concentration of ethanol: 1%). In all assays, two negative and two positive controls were used. Negative controls: (i) cells in DMEM (control 1) and (ii) cells in DMEM and ethanol (final concentration of ethanol: 1%; control 2). Positive controls: 17β-estradiol (estrogen, Sigma-Aldrich, Germany) and tamoxifen (anti-estrogen, Sigma-Aldrich, Germany). Both positive controls were performed to show the accuracy of the tests and the usual behaviour of the cell cultures (data not shown). MTT test: After incubation with MTT for 4 h at 37°C and 5% CO<sub>2</sub>, the solvent was added and the plates were incubated overnight. The absorbance of the formazan crystals was measured at 570 nm (reference wavelength 670 nm) by using a microplate ELISA reader (BioRad, Hercules, CA, USA).

BrdU test: After labelling with BrdU for 3 h, the cells were fixed and BrdU incorporation into DNA was measured at 450 nm (reference wavelength 620 nm).

LDH test: The LDH activity of the supernatants was measured at 492 nm (reference wavelength 620 nm). Additionally, a Triton X-100 control was added at a final concentration of 1% for the determination of the maximum release of LDH activity (100%).

### Flow cytometry

MCF7 cells ( $5 \times 10^5$ /ml) were grown for 24 h in 6-well-plates. After



**Figure 1.** Pyrolysis Field Ionisation Mass Spectrometry: Data (mean  $\pm$  SE) (standard error) represent the relative abundance of the total ion intensity (% TII) of 12 compound classes in leaf, stem and root extracts from flax (*Linum usitatissimum*). Asterisks (\*) indicate significant differences between the extracts of the plant samples. CHYDR: carbohydrates; PHLM: monolignols; LDIM: lignin dimers; LIPID: alkanes, alkenes, aldehydes, alcohols, fatty acids, n-alkyl ester, waxes, fats; NCOMP: N-containing compounds (mainly heterocyclic); ISOPR: isoprenoid compounds (sterols, terpenes, carotinoids); PEPTI: peptides and free amino acids; LOWMW: low molecular compounds; LIGNA: lignans; FLAVO: flavones; ISOFL: isoflavones; POLYO: other polyphenols (for example, suberins, cutins, stilbenes).

the exchange of the medium, only root extract (0.01, 50 and 500  $\mu\text{g/ml}$ ) or tamoxifen (50  $\mu\text{g/ml}$ ) were added and incubated for 24 h at 37°C and 5%  $\text{CO}_2$ . Cells were washed with phosphate-buffered saline (PBS), treated with trypsin, centrifuged and washed again. They were treated with 1 mg/ml RNase (Sigma-Aldrich, Germany) at 37°C for 20 min and incubated with propidium iodide (50  $\mu\text{g/ml}$ , Sigma-Aldrich, Germany) for 3 h on ice. Flow cytometry was performed with BD FACSCalibur, equipped with an argon-ion laser of wavelength 488 nm (BD Bioscience). For data acquisition, CellQuest Pro 4.0.1 (BD Bioscience) was used.

### Statistical analysis

Statistical analysis was performed by using the Student's *t*-test for a comparison of the means. Data are presented as mean  $\pm$  standard deviation (SD).  $P < 0.01$  was considered as being statistically significant and is denoted by an asterisk.

## RESULTS

### Pyrolysis field ionisation mass spectrometry

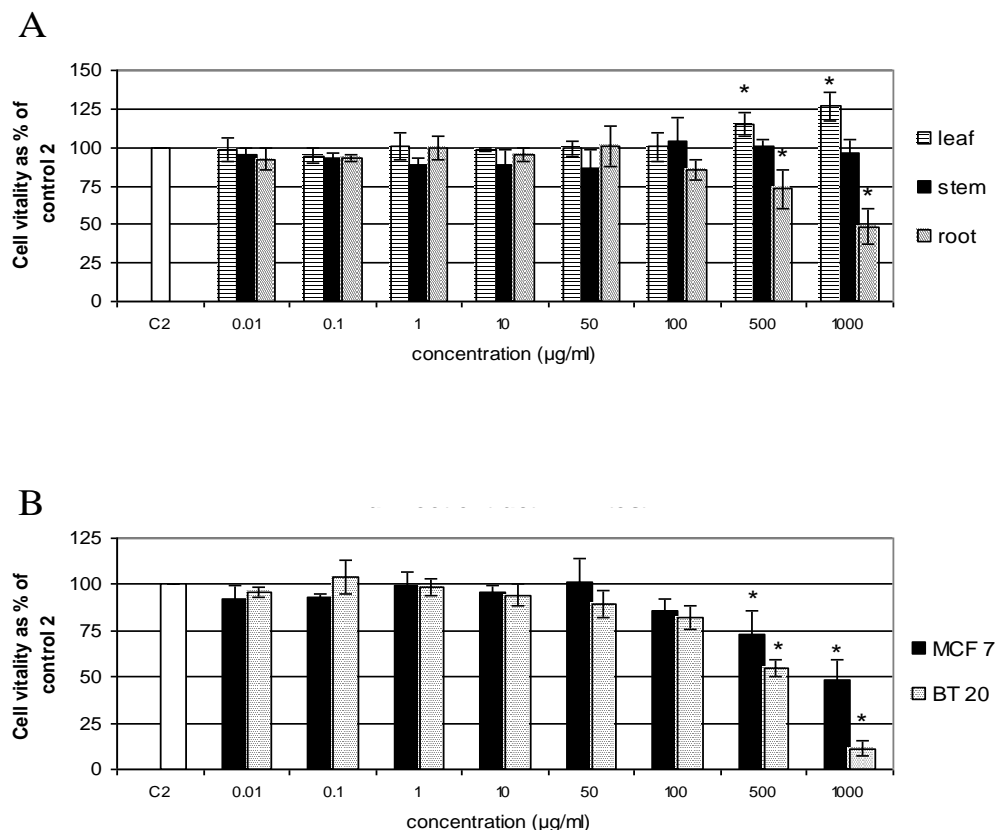
The molecular-chemical screening of leaf, stem and root ethanolic extracts from *L. usitatissimum* by Py-FIMS revealed a broad mass signal pattern with 429, 374 and 442  $m/z$  signals (data not shown). The signals were higher than 0.01% of total ion intensity (TII) for leaf, stem and root extract from the flax plant, respectively. The bulk data of summed and averaged mass spectra was condensed to 12 compound classes. The relative abundance of TII is visualised in Figure 1, where obvious differences can be observed between the various plant extracts. Thus, significantly higher percentages of TII

were found in root extracts of *L. usitatissimum* compared with those of the leaf and stem for the 8 compound classes monolignols (PHLM), lignin dimers (LDIM), alkanes (LIPID etc.), N-containing compounds (NCOMP), lignans (LIGNA), flavones (FLAVO), isoflavones (ISOFL) and other polyphenolics (POLYO). These compound classes, with the exception of LIPID, showed up to three times higher relative abundances in the root extract compared with those of the leaf or stem extract (Figure 1).

For the LIPID class, including alkanes, alkenes, n-alkyl ester, aldehydes, alcohols, fatty acids and waxes, the difference of the percentage of TII between the root, the leaf and the stem extract was negligible. The largest difference of TII was determined for the PHLM: about 12% for the root extract versus 2 and 6% for the leaf and stem extract, respectively. POLYO was also twice as high in the root versus the leaf and stem extracts. For isoprenoids (sterols, terpenes, carotinoids), the TIIs were ranked in the reverse order. This compound class dominated in leaf (50%) and stem (41%) extracts, but was low in root extract (17%). The compound classes of carbohydrates, peptides and low molecular compounds were identified both with low TII and without significant differences between root, leaf and stem extracts.

### Influence of flax extracts on cell vitality

In MCF7 cell lines, low concentrations of leaf, stem and root extracts (0.01 to 100  $\mu\text{g/ml}$ ) did not affect cell vitality. However, high extract concentrations (500 and 1000  $\mu\text{g/ml}$ ) activated (leaf extract) or inhibited (root extract)



**Figure 2.** Effect of various concentrations of leaf, stem and root extracts from flax (*Linum usitatissimum*) on the cell vitality (MTT test) of the MCF7 cell line (A) and the effect of various concentrations of flax root extracts on the cell vitality of the MCF7 and BT20 cell lines (B). Data (mean  $\pm$  SD) represent the relative formation of formazan from MTT in % in comparison with negative control 2 (100%) as obtained in at least 3 experiments. Asterisks (\*) indicate significant differences between treated cells and the negative control 2 ( $P < 0.01$ ). C2: negative control 2, cells treated with ethanol (final concentration 1%).

cell activity significantly; a mean inhibition of 50% was measured with 1000 µg/ml (Figure 2A). While flax root extract concentrations between 0.01 and 100 µg/ml did not affect the vitality of BT20 cells, 500 and 1000 µg/ml were very effective. Compared to MCF7 cells, BT20 cells were much stronger inhibited (up to 90% at 1000 µg/ml) (Figure 2B). The values of the two negative controls of both cell types did not differ, indicating that 1% ethanol did not inhibit the growth of MCF7 and BT20 cells (data not shown).

#### Influence of flax extracts on cell proliferation

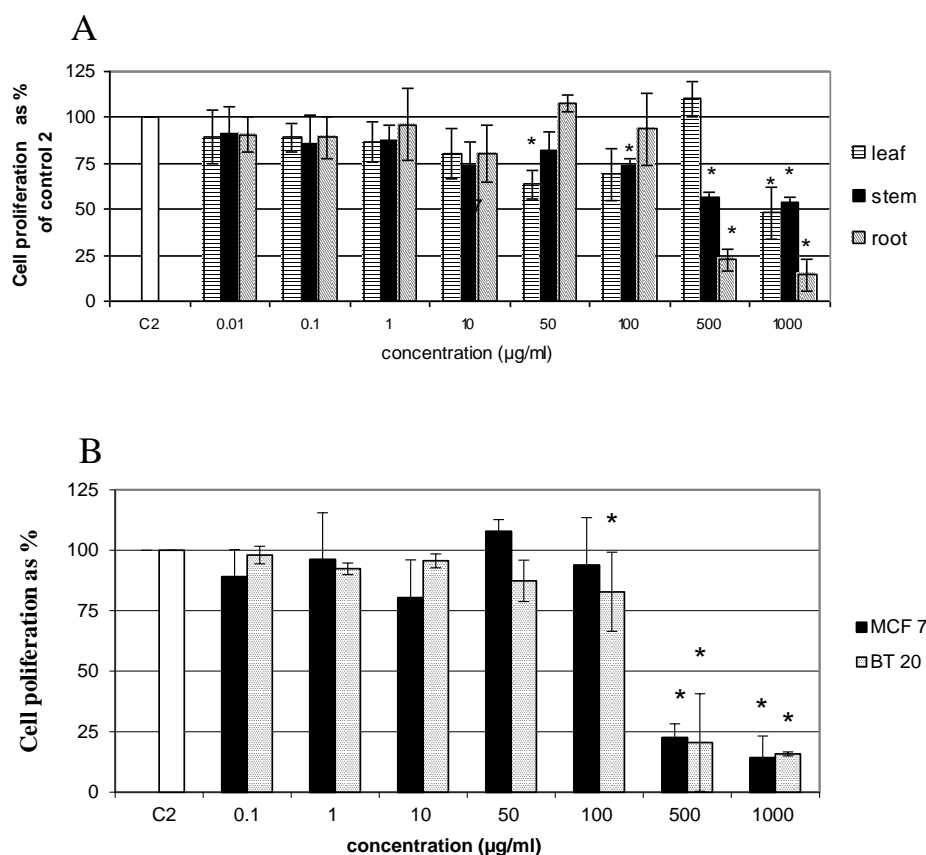
Leaf, stem and root extracts did not affect MCF7 cell proliferation (BrdU test) at low concentrations (0.01 to 10 µg/ml) (Figure 3A). At 50 to 500 µg/ml, the leaf extract induced the activation or inhibition of cell proliferation, at 1000 µg/ml inhibition was about 50%. The stem extract was weakly inhibitory at 100 µg/ml, concentrations of 500 and 1000 µg/ml inhibited cell proliferation by 25 to 40%.

At concentrations of 500 and 1000 µg/ml, root extract showed the strongest inhibitory action (up to 85%) of all extracts. Flax root extract concentrations between 0.01 and 50 µg/ml did not influence the growth of BT20 cells, but 100, 500 and 1000 µg/ml inhibited cell proliferation up to 85% (Figure 3B).

#### Effect of flax extracts on cytotoxicity in MCF7 and BT20 cell lines

Low concentrations (0.01 to 10 µg/ml) of flax leaf, stem and root extracts did not induce cytotoxic effects in MCF7 cell lines (LDH test) (Figure 4A). Higher concentrations of the leaf and stem extracts ( $> 50$  µg/ml) were dose-dependently cytotoxic. In contrast, the whole range of flax root extract concentrations produced only low cytotoxic effects on MCF7 cells (Figure 4B). However, in BT20 cell lines, the lethality reached 60 to 100% after the addition of high concentrations of flax root extract (100 to 1000 µg/ml) (Figure 4B).





**Figure 3.** Effect of various concentrations of leaf, stem and root extracts from flax (*Linum usitatissimum*) on the cell proliferation (BrdU test) of the MCF7 cell line (A) and the effect of various concentrations of flax root extracts on the cell proliferation of the MCF7 and BT20 cell lines (B). Data (mean  $\pm$  SD) represent relative BrdU uptake in % in comparison with negative control 2 (100%) as obtained in at least 3 experiments. Asterisks (\*) indicate significant differences between treated cells and the negative control 2 ( $P < 0.01$ ). C2: negative control 2, cells treated with ethanol (final concentration 1%).

### Induction of apoptosis

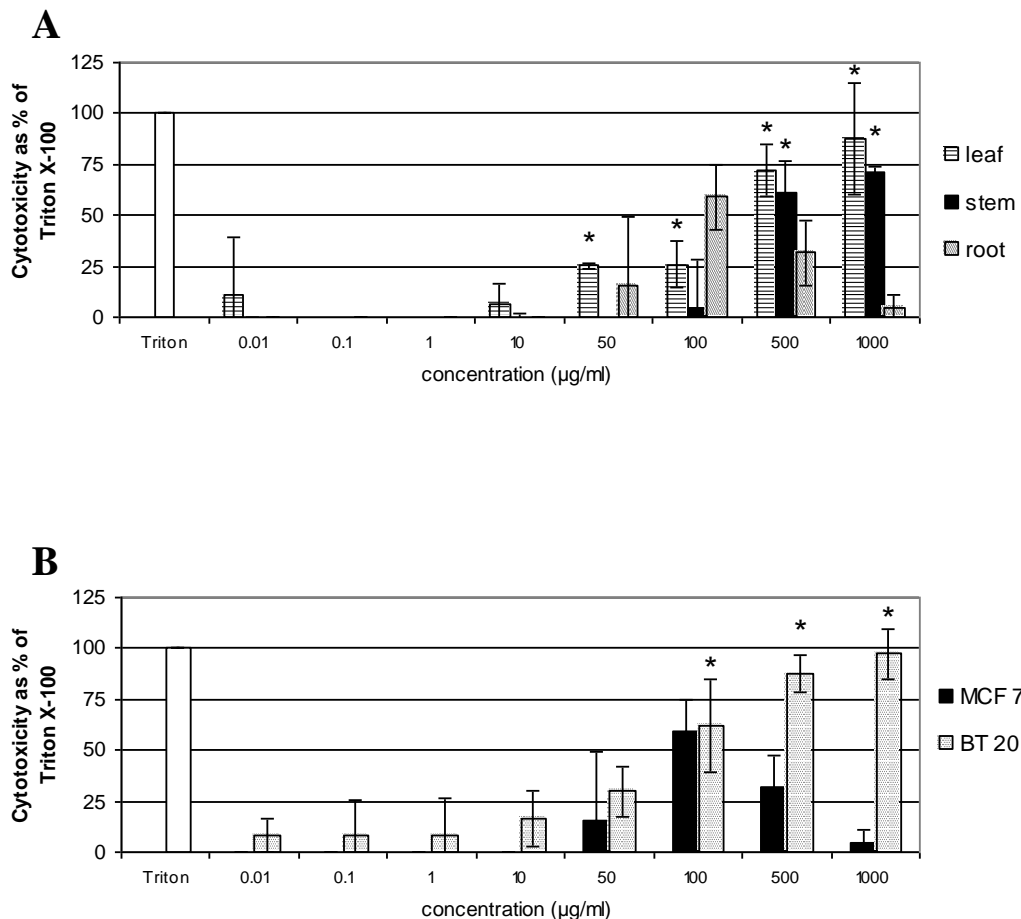
Induction of apoptosis was tested in MCF7 cells. The apoptotic potency of flax root extracts in MCF7 cells was analysed by flow cytometry (Figure 5). Only the high concentration (500 µg/ml extract) resulted in a significant increase of apoptosis (about 25%). For comparison, tamoxifen at 50 µg/ml resulted in 35% apoptosis of the cells.

### DISCUSSION

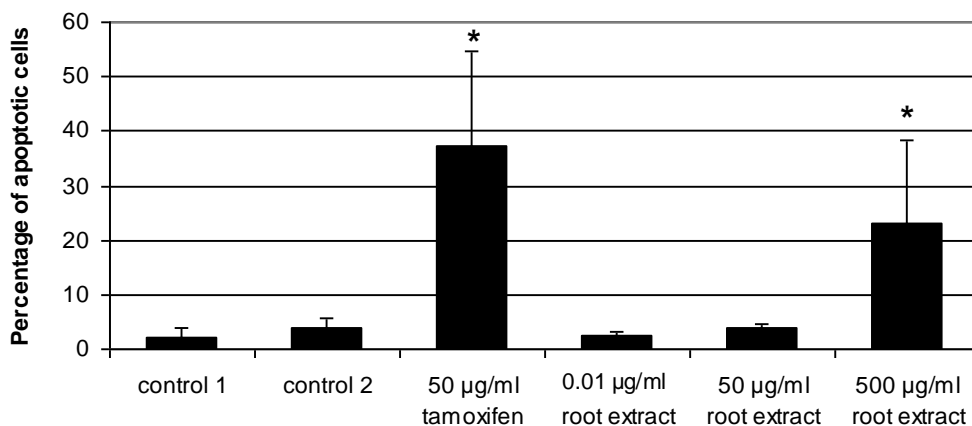
The major classes of phytoestrogens, the isoflavones and lignans, are found at high levels in soybean, flax seed and in various other parts of plants (Kulling and Watzl, 2003; Rickard and Thompson, 1997). Inhibitory effects of isolated isoflavones and lignans on breast and colon cancer were reported in several studies (Rickard-Bon and

Thompson, 2003; Zaizen et al., 2000). However, in this context the different roles of receptor-dependent and independent mechanisms of phytoestrogens are still only poorly investigated. We have therefore embarked on a systematic investigation to test the influence of extracts from three parts of *L. usitatissimum* on human mammary carcinoma cell lines *in vitro*. We have exposed human ER-positive (MCF7) and ER-negative (BT20) breast cancer cell lines to flax extracts. Different responses indirectly could provide information on the involvement of ERs and of phytoestrogens.

High-pressure liquid chromatography (HPLC)-MS analysis has shown by using various extraction methods that root, stem and leaf extracts of *L. usitatissimum* contain more representatives from the lignan domain (for example, secoisolariciresinol, matairesinol, arctigenin) than isoflavones (for example, genistein, daidzein) (Abarzua et al., 2007). In the present study, Py-FIMS has provided evidence that flax root extracts are especially



**Figure 4.** Effect of various concentrations of leaf, stem and root extracts from flax (*Linum usitatissimum*) on cytotoxicity (LDH test) in the MCF7 cell lines (A) and the effect of various concentrations of flax root extracts on cytotoxicity in the MCF7 and BT20 cell lines (B). Data (mean ± SD) represent relative cytotoxicity in % in comparison with negative control 2 and Triton X-100 control (100 %) obtained in at least 3 experiments. Asterisks (\*) indicate significant differences between treated cells and the negative control 2 ( $P < 0.01$ ). Triton: Triton X-100 control.



**Figure 5.** Effect of various concentrations of root extracts from flax (*Linum usitatissimum*) (0.01, 50, 500 µg/ml) and of 50 µg/ml tamoxifen on the percentage of apoptotic MCF7 cells. Data (mean ± SD) represent relative apoptosis in % obtained in at least 3 experiments. Asterisks (\*) indicate significant differences between treated cells and the negative control 2 ( $P < 0.01$ ); control 1: untreated cells, control 2: cells treated with ethanol (final concentration 1%).

rich in polyphenols and monolignols (Figure 1), that is contain large amounts (21%) of polyphenols, including lignin dimers, flavones, isoflavones, lignans and related compounds such as suberins, cutins and stilbenes, whereas in leaf and stem extracts they represent less than 9% of all compounds.

Plant cell walls containing high amounts of polyphenols such as suberins and lignins have been suggested as the most likely dietary components being protective against cancer (dietary fiber hypothesis) (Ferguson et al., 2001). Several studies have documented the anti-proliferative and cytotoxic effects of isolated lignans (Awale et al., 2006; Moritani et al., 1996; Singh et al., 2007). As in the present study the flax root extract induced stronger inhibitory effects on cell vitality and proliferation of human breast cancer cells *in vitro* than leaf and stem extracts (Figures 2A and 3), we suppose that phenolic compounds, for example isoflavones, lignans and monolignols, play an important role in the inhibitory effects. Isoflavones (for example, genistein, daidzein, equol) can alter the expression of genes that are important for cell survival (Moiseeva et al., 2007) and cell cycle (Touny and Banerjee, 2006). They suppress NF $\kappa$ B (Li and Sarkar, 2002) and induce caspase-mediated apoptosis (Casp 7, 9) (Charalambous et al., 2013). For genistein, an increased expression of tumor suppressor genes p21 and p16 and a decreased expression of the tumor promoting genes BMI1 and c-MYC is described (Li et al., 2013).

The cell vitality of ER-positive and ER-negative human breast cancer cell cultures was not affected by low concentrations of flax root extract (0.01 to 100  $\mu$ g/ml). Significant inhibition in a dose-dependent manner became obvious only after the addition of high concentrations (>100  $\mu$ g/ml) (Figure 2B). The inhibition of cell vitality by flax root extracts is stronger in the ER-negative cell line BT20 than in the ER-positive MCF7 (Figure 2B). These differences suggest that flax root extracts can affect the growth of the cell lines by both ER-mediated and ER-independent mechanisms of action.

However, the differences may also indicate a higher sensitivity for flax root extracts in the ER-negative BT20 cell lines compared to the ER-positive MCF7 cells. A former study demonstrated an inhibition of cell growth of ER-positive and -negative cell cultures after the application of genistein at IC<sub>50</sub> values from 6.5 to 12.0  $\mu$ g/ml (Peterson and Barnes, 1991). An extract from *Cimicifuga racemosa* reduced the cell proliferation and enhanced the rate of apoptosis in several human breast cancer cell lines, independently of their ER status (Hostanska et al., 2004).

The flax leaf and stem extracts were more cytotoxic than the root extracts (Figure 4A). Leaf extract, in particular, induced an increase of the markers of cell proliferation and vitality at concentrations of 500 and 1000  $\mu$ g/ml. As only about 25% of MCF7 cells survived the addition of leaf extract, this must reflect an enormous

increase of enzymatic activity in the surviving cells. The increased viability of MCF7 cells after the addition of high concentrations of leaf extracts might be attributable to higher energy-consuming activities before death. Similar observations have been reported for soybean isoflavones (Zhou et al., 1998). Moorghen et al. (1998) have suggested that cells try to compensate for an increased apoptotic rate by an increase in cell proliferation.

With regard to the cytotoxic potential of the flax root extract, the ER status of the cells seems to be important. Whereas the cytotoxicity in BT20 cells increased with rising concentrations of flax root extract in a dose-dependent manner, in the MCF7 cell line addition of lower concentrations such as 50 and 100  $\mu$ g/ml extract increased cytotoxicity, but higher concentrations decreased cytotoxicity (Figure 4B). About 90 or 98% of the BT20 cells did not survive the application of 500 or 1000  $\mu$ g/ml root extract, respectively (Figure 4B). This correlates with the strong inhibition of cell vitality of the BT20 cells after the addition of 500 and 1000  $\mu$ g/ml root extract (Figure 2B). An addition of root extract at 500  $\mu$ g/ml caused the death of about 30% of MCF7 cells (Figure 4A). This result is supported by flow cytometric analysis, showing a percentage of 25% apoptotic cells after the application of the 500  $\mu$ g/ml root extract (Figure 5).

These results confirm data of changes of cell adhesion recorded with the Bionas® 2500 analyzing system after the addition of low (0.01, 1, 50  $\mu$ g/ml) and high (100, 200, 1000  $\mu$ g/ml) concentrations of root extracts of *L. usitatissimum* (Abarzua et al., 2010). Low concentrations did not influence adhesion in MCF7 cells, whereas high concentrations resulted in dramatic morphological inhibition. Several studies have described the induction of apoptosis as a response to phytoestrogens. Jo et al. (2005) and Danbara et al. (2005) have shown that the induction of apoptosis is caused by the "human lignan" enterolactone as a result of the metabolism of plant lignans in their experiments. We therefore suggest that the apoptosis of MCF7 cells might be induced by the phenols and monolignols detected in the root extract of *L. usitatissimum* (Figure 1). In spite of the low cytotoxicity with regard to MCF7 cells, after the addition of 500  $\mu$ g/ml of root extract significant inhibition of cell vitality and proliferation occurred in up to 25 and 75%, respectively (Figure 2A and 3).

Since the root extract of *L. usitatissimum* induced a significant inhibition of cell vitality and proliferation without incurring strong cytotoxicity in the human mammary carcinoma cell line MCF7, which might qualify the extract for prevention of hormone sensitive carcinomas, it seems worthwhile to further characterize the presumable phytoestrogens in the extract and the relevant intracellular processes, in which they are involved. At the moment our investigations resulted in an extract that might possibly be used as supplement for therapy or prevention in future. For this case it is necessary to study the reactions

of normal mamma epithelial cells to addition of flax root extract in further investigations.

## ACKNOWLEDGEMENTS

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Full Length Research Paper

## Water extracts of dietary mushrooms, *Agrocybe aegerita* and *Hypsizigus mamoreus*, inhibit antigen expression of human hepatitis B virus

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Hepatitis B virus (HBV) infection is prevalent but the current treatments remain to be improved. This study evaluated the effect of dietary mushrooms on expression of surface (HBsAg) and e (HBeAg) antigens of HBV. Hot-water extracts (HWE) of dietary mushrooms, *Agrocybe aegerita* (AA), *Hypsizigus mamoreus* (HM) and *Flammulina velutipes* (FV), were prepared and subjected to gel permeation chromatograph for molecular weight separation. Human hepatoma cell lines Hep3B and Huh7 harboring HBV genome were used as an *in vitro* model for expression of HBsAg and HBeAg. Primary cultured mouse hepatocytes were used as a normal counterpart. HWE of AA and HM, but not of FV, possessed significant inhibitory activity against the expression of HBsAg in Hep3B and Huh7 cells. The activity of high molecular weight fraction of AA and HM was greater than the lower ones. Furthermore, HWE of AA and HM inhibited the expression of HBeAg in Hep3B cells. The HWE of AA and HM did not affect cell viability of neither hepatoma cell lines nor primary normal hepatocytes. In conclusion, the hot-water extracts of dietary mushrooms *A. aegerita* and *H. mamoreus* could inhibit the antigen expression of human HBV in host cells without toxicity to normal primary hepatocytes.

**Key words:** Mushrooms, hepatitis B virus, HBsAg, HBeAg.

### INTRODUCTION

Hepatitis B virus (HBV) infection is regarded as a critical health issue with increasing prevalence. HBV infection is highly correlative to the development of liver cirrhosis and carcinogenesis of hepatocellular carcinoma, a malignancy with extremely poor prognosis. The current antiviral agents for HBV infection include lamivudine, adefovir,

entecavir, interferon (IFN)-alpha, etc (Cooke et al., 2010). However, the effectiveness of anti-viral agents, especially nucleoside analogs, is limited by drug resistance resulted from gene mutation of HBV after long-term administration (Papatheodoridis et al., 2008; Shaw et al., 2006). For example, administration of lamivudine results in high rates

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of resistance due to emergence of HBV strains with tyrosine-methionine-aspartate-aspartate (YMDD) mutation (Allen et al., 1998). Therapy of finite duration using IFN- $\alpha$  administration is frequently interrupted by its toxicity including pyrexia, fatigue, headache, myalgia, depression, and myelosuppression (Lau et al., 2005). These limitations compromise the therapeutic efficacy of current practice against HBV infection. Clearly, it is important to develop a novel category of therapeutics against HBV infection through different mechanisms of action. Since normal liver is the main organ responsible for detoxification, the development of anti-HBV agents from non-toxic food, such as dietary mushrooms, might be a legitimate strategy.

Cold-water extracts of dietary mushrooms, *Hypsizigus marmoreus* (HM), *Agrocybe aegerita* (AA) and *Flammulina velutipes* (FV), have been reported capable of stimulating cytokine secretion of mononuclear cells to inhibit growth of leukemia cells (Ou et al., 2005). The cytokines augmented in secretion from mononuclear cells included tumor necrosis factor-alpha and interleukin-1 beta. Of these cold-water extracts, the high molecular weight fractions possessed this activity greater than the low molecular weight counterparts. However, whether these bioactive dietary mushrooms can inhibit expression of viral proteins, including the HBsAg, remains to be determined.

The purpose of this study was to examine the anti-HBV effect of the commonly used dietary mushrooms. Expression of surface and e antigens of HBV in host cells was served as the major endpoint. Primary hepatocytes isolated from mice were used as normal counterpart for evaluation of hepatic toxicity.

## MATERIALS AND METHODS

### Preparation of extracts from mushrooms

Fresh dietary mushrooms of *A. aegerita* (AA), *H. marmoreus* (HM), or *F. Velutipes* (FV) were purchased from a supermarket with characterization by a qualified fungologist in the National Research Institute of Chinese Medicine, Taiwan. The edible portion was cleaned by washing 3 times in distilled water and was cut into pieces about 5 mm  $\times$  5 mm in size. In our preliminary work, we used solvents with various polarities, including water, ethyl acetate, methanol and n-hexane. It showed that water extract was the most active one on inhibiting expression of HBsAg. After initial screening of bioactivity against HBsAg expression, hot-water (65°C for 30 min) extracts (HWE) were determined more effective than cold-water (4°C for overnight) extracts. After centrifugation (12,000 g, 4°C, 30 min), the obtained supernatant was lyophilized for quantification for further experiments. The dissolved HWE was then fractionated by a Sephadex G-50 (molecular weight range 1.5 to 30 kDa) gel permeation chromatography (column size, 2.6 cm  $\times$  65 cm; eluent, distilled water; fractionation, 5 ml/tube) to pool the high (HF) and low (LF) molecular weight fractions under monitored at 280 nm. Lyophilized powders of HWE, HF and LF were dissolved and dialyzed against phosphate-buffered saline prior to use.

### Hepatoma cell culture

Human hepatocellular carcinoma cell lines Hep3B and Huh7 harboring endogenous and integrated HBV genome, respectively, with stable production of HBsAg, were used as the *in vitro* model for HBV replication (Knowles et al., 1980; Twist et al., 1981). These hepatoma cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum, 10<sup>5</sup> IU/L penicillin, 100 mg/L streptomycin, and 1 mmole/L L-glutamine, in a humidified 5% CO<sub>2</sub> incubator at 37°C.

### Preparation and culture of primary mouse hepatocytes

Primary hepatocytes were isolated from 6 to 8 week-old male Balb/c mice according to the procedures established by Klaunig et al. (1981), with modification. In brief, after anaesthetized and laparotomy, the portal vein was perfused with calcium-free Hanks balanced salt solution containing ethylene glycol-bis-(b-aminoethyl) N, N9-tetraacetic acid and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid. This was followed by perfusion with the same solution containing collagenase for isolation of hepatocytes. After removal of liver, hepatocytes were mechanically dissociated and filtered through mesh. Hepatocytes were isolated by gradient centrifugation with Percoll (GE Healthcare, Little Chalfont, UK) and then washed. Cells were further cultured and plated with complete medium and adequate hepatocyte growth factor. The medium was changed regularly as necessary.

### Growth Inhibition and cell viability by MTT assay

The cell viability of hepatoma cells was assessed by a tetrazolium dye colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) test (Mosmann, 1983) and expressed as - MTT value of experimental group/MTT value of untreated control group.

### Assay for relative HBsAg and HBeAg expression

Hepatoma cells cultured in DMEM with 10% fetal bovine serum for 24 h were transferred to serum-free DMEM with or without fungal extractions and incubated thereafter. The secreted HBsAg and HBeAg in the culture medium were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (General Biological, Taipei, Taiwan). The detection of HBsAg in serum of patients indicated a current HBV infection and the risk for developing compensated cirrhosis and hepatocellular carcinoma. Thus, the HBsAg is a useful index to evaluate the viral activity (Czaja, 1979). The relative HBsAg expression was determined by the following formula: (HBsAg / MTT) from treated cells / (HBsAg / MTT) from the untreated cells. The measurement of secreted viral antigens from HBV host cells, such as HBsAg secretion from Hep3B cells has been applied in various investigations to evaluate the effect of drugs on HBV activity. To further validate the anti-HBV effect of active fractions, the relative HBeAg expression was determined with similar methods.

### Statistical analysis

Results are presented as mean  $\pm$  standard error of mean (SEM). Differences between the treatment groups was assessed by Student's t-test. A confidence level of 5% ( $p < 0.05$ ) was considered significant.

**Table 1.** Bioactivity of high and low molecular weight fractions of mushrooms against expression of HBsAg.

Parameter	Relative expression of HBsAg (%)	
	Hep3B cell	Huh7 cell
<b>AA</b>		
HWE	36.6±3.2	52.9±5.4
HF	25.1±2.3*	33.5±3.6*
LF	39.4±2.9	57.3±4.3
<b>HM</b>		
HWE	41.3±5.8	63.1±3.8
HF	32.7±3.3*	44.8±4.5*
LF	48.6±3.1	60.7±2.7

Hep3B and Huh7 cells were treated with hot water extracts (HWE), their high molecular weight fraction (HF) or low molecular weight fraction (LF) of mushrooms at concentration of 50 µg/ml for 48 h and then subjected to ELISA. Triplicated data from separate experiments are expressed as mean ± SEM. \* $p < 0.05$  for HF versus LF. AA: *Agrocybe aegerita*; HM, *Hypsizigus mamoreus*.

## RESULTS

### Effect of mushroom extracts on viability of hepatoma cells

HWE of AA, HM and FV at concentrations from 0 to 400 µg/ml did not significantly affect the cell viability of Hep3B and Huh7 cells (Figure 1A and B). Upon this basis, the biological activity of mushroom extracts in these cells could be considered not owing to cellular toxicity.

### Reduction of relative HBsAg and HBeAg expression in hepatoma cells by mushroom extracts

Lamivudine, as a positive control, caused  $83.9 \pm 5.6\%$  reduction in HBsAg expression at 25 µM. As shown in Figure 2A and B, HWE of AA, HM, but not FV, suppressed both the endogenously expressed HBsAg in Hep3B cells and the HBsAg produced from the stable clone of HBV DNA-integrated in human hepatoma Huh7 cells. This inhibitory effect was concentration-dependent up to  $78.2 \pm 4.1$  and  $71.3 \pm 3.7\%$  reduction in Hep3B and Huh7 cells, respectively. The estimated 50% HBsAg-inhibitory concentrations ( $IC_{50}$ ) of AA and HM on Hep3B cells was 22.5 and 37.4 µg/ml, respectively. Further isolation and assessment of higher and lower molecular weight fractions from HWE of AA and HM demonstrated a greater HBsAg-inhibitory activity by the higher ones (Table 1). Furthermore, the HWE (50µg/ml) of AA and HM inhibited the expression of HBeAg by  $70.3 \pm 4.7$  and  $61.8 \pm 5.1\%$  in Hep3B cells, respectively (Figure 3).

### Effect of mushrooms extracts on viability of normal hepatocytes

Primarily cultured murine hepatocytes were served as a normal counterpart to assess the normal liver toxicity of mushroom extracts. As demonstrated in Figure 4, HWE from AA and HM, at the concentrations given for inhibiting HBsAg expression in hepatoma cells, did not significantly reduce the viability of normal hepatocytes.

## DISCUSSION

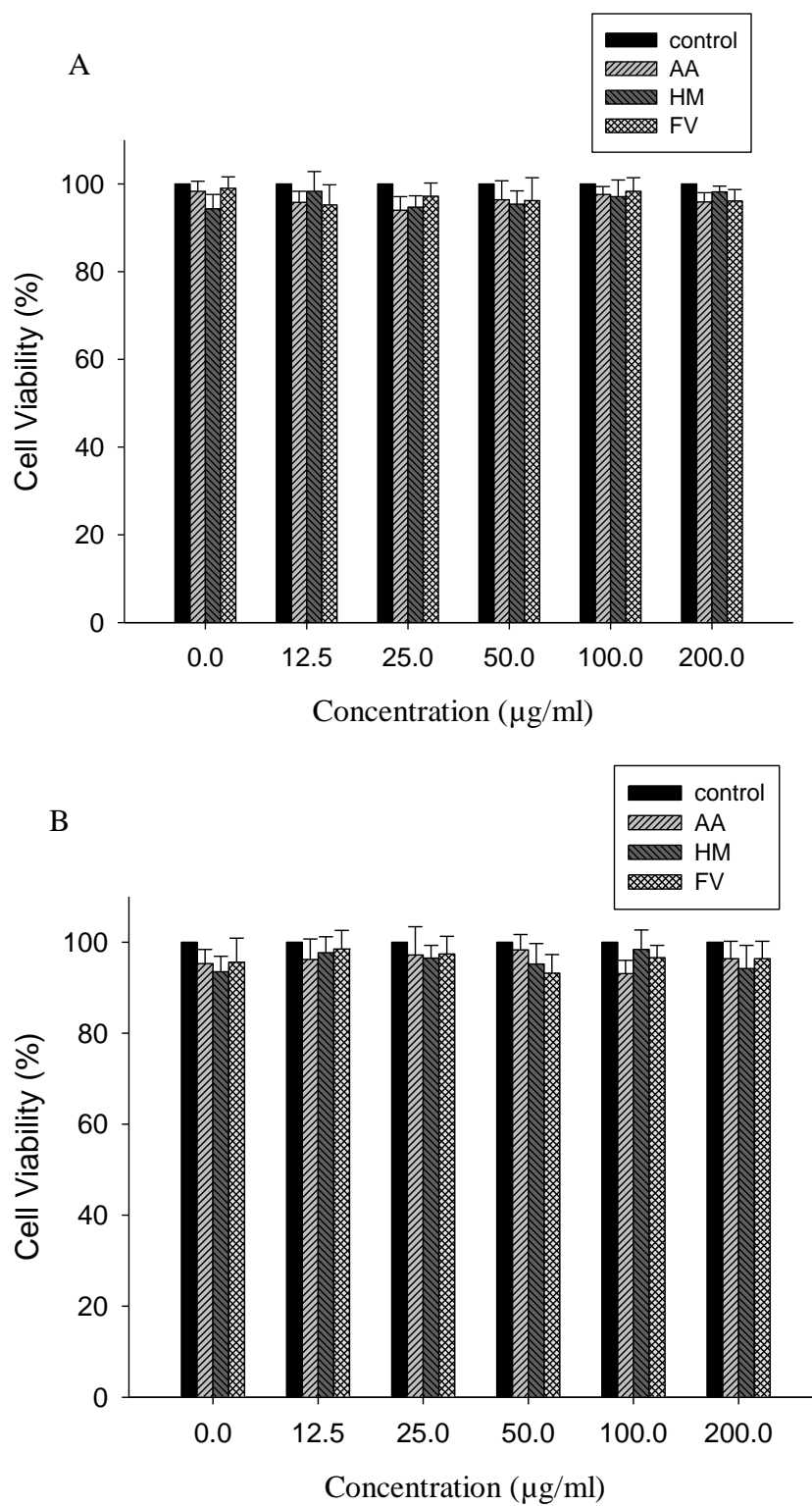
We found that hot water extracts of dietary mushrooms AA and HM inhibited the expression of human HBsAg and HBeAg in host cells without toxicity to normal primary hepatocytes. As mentioned before, the current treatments against HBV are either toxic or prone to be resisted by gene mutation. Our finding that the hot water extracts resembling cooking processes of two dietary mushrooms could inhibit the expression of HBsAg and HBeAg without toxicity to hepatocytes. This may shed a light for development of new therapeutic agents against HBV from food. Whether the bioactivity of mushroom extracts could be preserved or not in gastrointestinal tract after digestion remains unclear. Further *in vivo* tests are definitively necessary.

The experimental model used in this study includes Hep3B cells harboring endogenous HBsAg and the stable clone of HBV DNA-integrated Huh7 cells. HWE of AA and HM inhibited HBsAg expression in both cell lines, indicating a universal activity against HBsAg expression. However, this experimental model only provides a screening platform for anti-HBV agents (Chen et al 1997). To draw the conclusion that AA and HM can inhibit HBV. Further molecular studies including that for viral replication need to be examined. The other endpoints for assessment of HBV activity, including mRNA expression of HBsAg and expression of HBV DNA, could also be used as indicator of HBV activity.

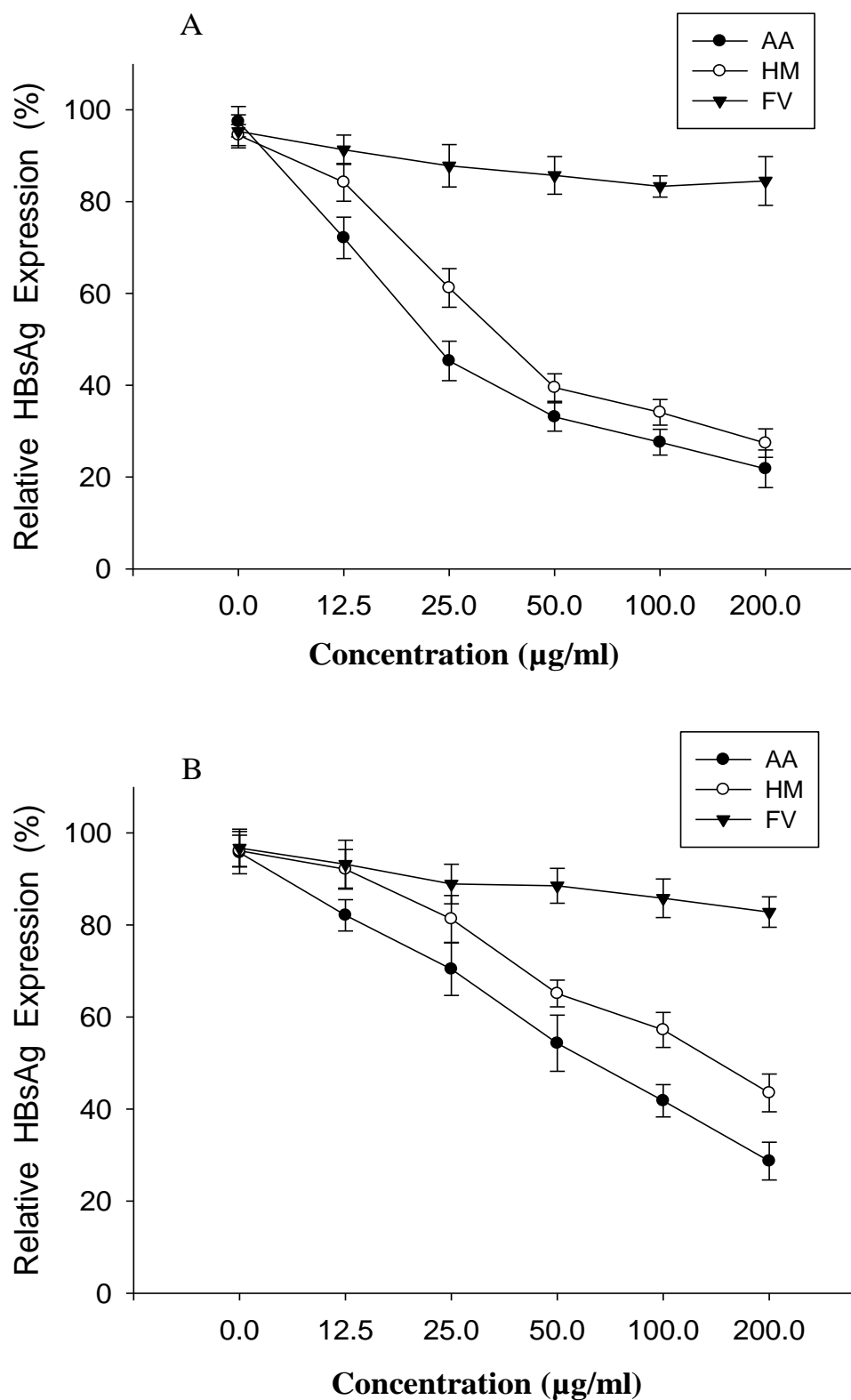
Normal liver toxicity assessed by *in vitro* hepatocytes viability shows no evident hepatotoxicity of mushroom extracts. Compared with the current anti-HBV therapeutics with various kinds of toxicity (Lau et al 2005), this further augments the potential of mushroom extracts to be developed as novel anti-HBV agents.

In clinical practice, chemotherapy-related HBV reactivation and resultant fulminate failure is not rare in HBV-infected patients (Hoofnagle, 2009; Yeo et al., 2000). Prophylactic administration of anti-viral agents such as lamivudine (Hsu et al., 2008) has been proved effective. Given that dietary mushrooms are safe and commonly used food, it is expected that AA and HM could be another option for prophylaxis of HBV reactivation in cancer patients whom already are heavily treated by chemotherapeutics.

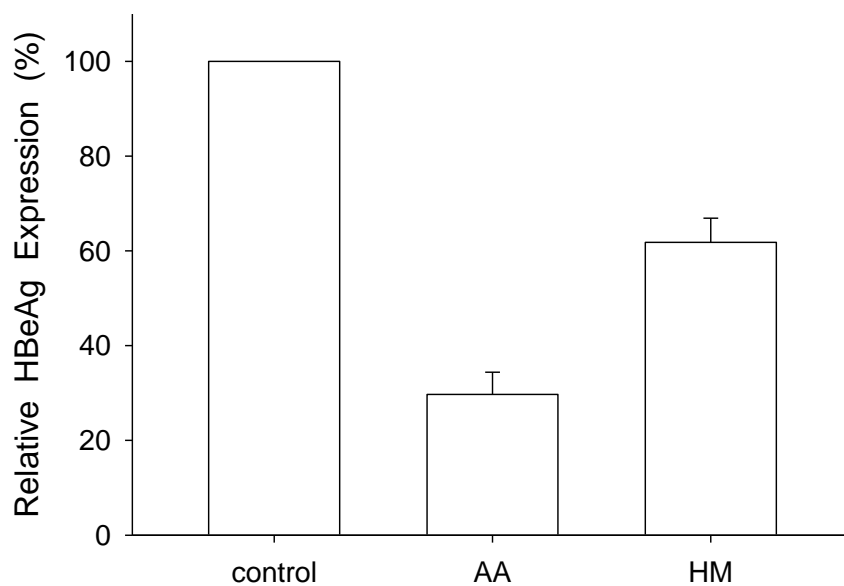




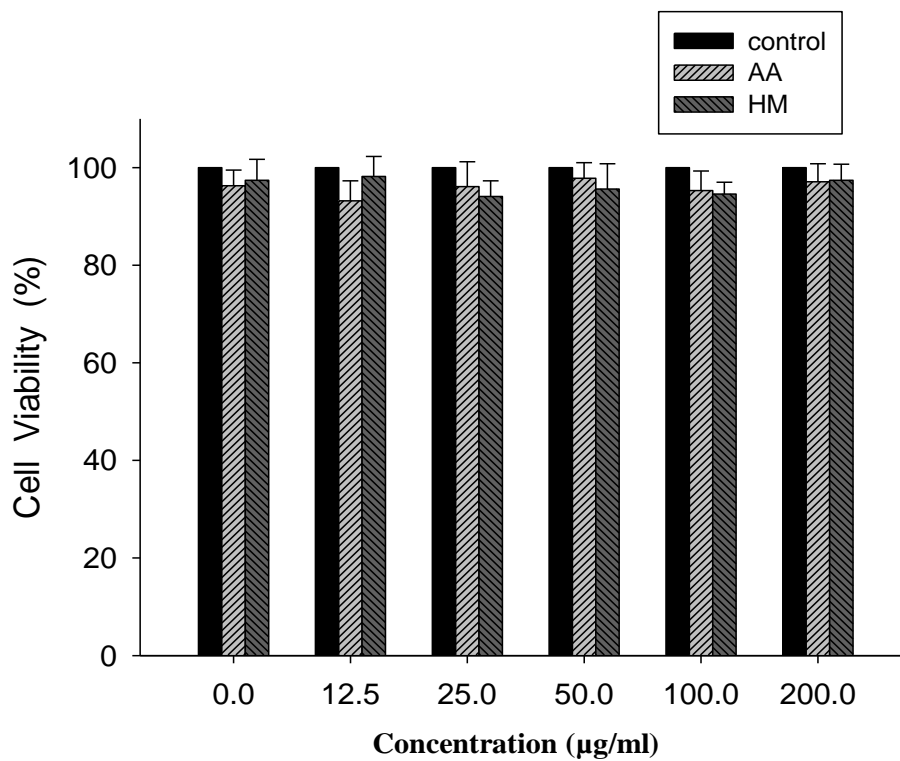
**Figure 1.** The viability of Hep3B and Huh7 hepatoma cells with treatment by extracts of mushrooms. A, Hep3B; B, Huh7 cells. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to MTT assay. Triplicated data from separate experiments are expressed as mean  $\pm$  SEM.



**Figure 2.** The relative HBsAg expression in Hep3B and Huh7 hepatoma cells. (A) Hep3B; (B) Huh7 cells. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to ELISA. Triplicated data from separate experiments are expressed as mean  $\pm$  SEM.



**Figure 3.** The relative HBeAg expression in Hep3B hepatoma cells. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to ELISA. Triplicated data from separate experiments are expressed as mean  $\pm$  SEM.



**Figure 4.** The viability of primary cultured hepatocytes with treatment by extracts of mushrooms. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to MTT assay. Triplicated data from separate experiments are expressed as mean  $\pm$  SEM.

## Conclusion

Hot water extracts of dietary mushrooms, *A. aegerita* (AA) and *H. mamoreus* (HM), could inhibit the antigen expression of HBV in host cells without toxicity to normal hepatocytes.

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Full Length Research Paper

## ***Polygonatum cirrhifolium* Royle and *Polygonatum verticillatum* (L.) Allioni: Status assessment and medicinal uses in Uttarakhand, India**

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***Polygonatum verticillatum* (Linn) All. and *Polygonatum cirrhifolium* (Wall.) Royle (Liliaceae) growing in the Himalayan region is assigned as vulnerable by International Union for Conservation of Nature and Natural Resources (IUCN). To elucidate the status of the plants in the Uttarakhand Himalaya population assessment of the species is done in the study region. The study area is divided into five sites (Kilburry, Jageshwar, Dunagiri, Chaubattia and Aboot mount) in Kumaun division and five sites (Bhavisya Badri, Tangnath, Dayara, Bharsar and Binsor) in Garhwal division. Population is assessed based on frequency, density, abundance and importance value index (IVI) of the plants in per square meter area following Mishra (1986). Threat assessment of species was done through six parameters (that is, habitat preference, distribution range, population size, use pattern, extraction trend and native area. Ethno-medicinal uses of the plants are also documented through interview and gathering with local informers/folk healers. Some conservation strategies are also suggested.**

**Key words:** International Health Regulations (IHR), population study, status, quadrat, sampling.

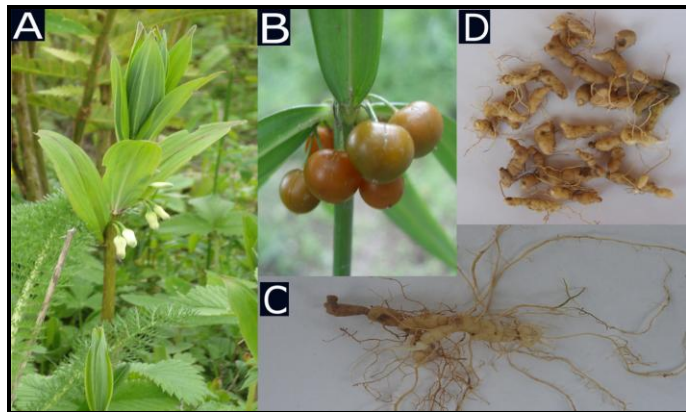
### INTRODUCTION

India possesses the world's richest medicinal plant heritage, traditional and local knowledge, and Himalaya is one of the mega biodiversity regions of the world (Heywood, 2000). In recent years, increasing attention is being paid to medicinal plant diversity due to their economic and conservation concern (Dhar et al., 2000).

It has been envisaged earlier that a number of medicinal plants of high importance are depleting at alarming rate (Tewari and Bhattacharjee, 1975). Unscientific harvesting, overexploitation of rhizome and other parts for medicinal use and consequent degradation of natural habitat along with the revival of traditional medicinal system in India and abroad, has put extra pressure on the forests, especially the medicinal plants. At least 90% of the plant species used in herbal industry today is extracted

from temperate to alpine zones of the Himalaya. Some important species which are traced directly from wild habitats are: *Aconitum heterophyllum*, *Picrorhiza kurrooa*, *Nardostachys grandiflora*, *Dactylorhiza hatagirea*, *podophyllum hexandrum*, *Polygonatum verticillatum*, *Polygonatum cirrhifolium*, *Berberis* Sp., *Paris polyphylla*. Many species of Himalayan medicinal plants are considered as most endangered and listed in Red Data Book of Indian plants (Nayar and Sastary, 1987, 1988, 1990). In the aforesaid circumstances, their *in-situ* conservation and *ex-situ* production appears to be the only remedy. Quantitative information on the microhabitats preferences and population dynamics are lacking from the region. Studies on these aspects of commercially important plants would be very vital in developing the conservation

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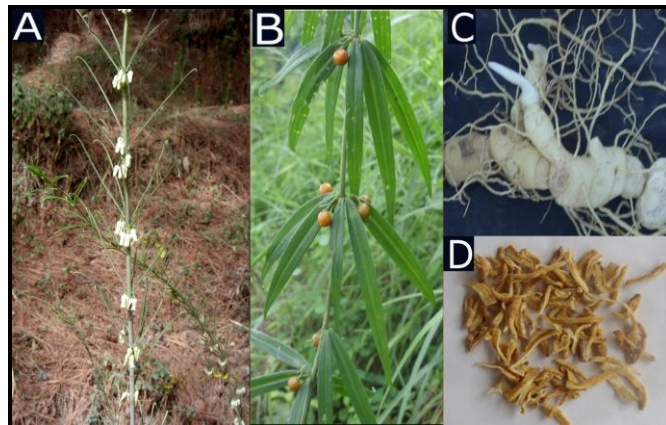


**Plate 1.** *Polygonatum verticillatum* (Linn.) All: (A) Live plant, (B) Live plant with bearing seeds, (C) Fresh root, (D) Dried root.

strategies for the area.

*Polygonatum* is a genus of erect or decumbent perennial herbs belonging to family Liliaceae and distributed in the temperate regions of the northern hemisphere. Thick fleshy creeping sympodial rhizomes characterize the genus. According to Miller (1754) the generic name of *Polygonatum* is derived from the character of the rhizome which resembles much as yovi, a Knee, because it has many little Knees. Linnaeus (1753) listed three species of *Polygonatum* under the genus *Convallaria*, namely, *Convallaria verticillata*, *Convallaria polygonatum* and *Convallaria multiflora* in his book '*Species Plantarum*'. These were treated under the generic name *Polygonatum* by Alloni (1785). In the natural system of classification of Angiosperms (Bentham and Hooker, 1862, 1883) family Liliaceae was classified in the series Coronarieae. *Polygonatum* is represented by 57 species in the world concentrated in Himalayas (Ohara et al., 2007). Out of the species occurring in Indian Himalayan Region (IHR), two (*P. verticillatum* (Linn.) All., and *P. cirrhifolium* (Wall.) Royle) are imperative ingredients of *Asthaverga*.

*P. verticillatum* (Linn.) All. syn. *C. verticillata* Linn., is known as whorled Solomon's seal in English and locally known as mitha dudhia (Nautiyal and Nautiyal, 2004) and Kantula (Gaur, 1999). The species is recognized as 'mahameda' in Ayurveda and in Sanskrit as Tridanti, Devamani and Vasuchhidra. It is an erect tall herb, 60 to 120 cm high. Leaves are whorled, sessile, 10 to 20 cm long, linear or lanceolate, acute or rarely tip carinate, glaucous beneath, occasionally ciliolate on margins and veins. Flowers are white, pinkish white or pale green, in whorled racemes, rarely lilac. The flowering and fruiting takes place in the month of June to October. This species is found in the temperate Himalayas at altitudes of 1800 to 3900 m (Plate 1). *P. cirrhifolium* (Wall.) Royle syn. *C. cirrhifolia* Wall is another member of *Asthaverga* recognized as King's Solomon's seal in English, locally as Khakan (Gaur, 1999), 'meda' in Ayurveda, Dhara,



**Plate 2.** *Polygonatum cirrhifolium* Royle: (A) Live plant, (B) Live plant with bearing seeds, (C) Fresh root, (D) Dried root.

Manichhidra and Svalpaparni in Sanskrit (Plate 2). It is also a tall erect, perennial herb, 60 to 120 cm high with whorled (3 to 6) sessile, linear leaves having tendril like tips. Flowers are white, green purplish or pink on short stocks and the fruits are round blue-black berry, when ripen found in the temperate Himalayas at the altitudes of 1200 to 4200 m. Rhizomes are thick and fleshy.

#### STUDY AREA

The state of Uttarakhand (28° 53' 24" and 31° 27' 50" N latitude and 73° 34' 27" and 81° 02' 22" E longitudes) includes parts of Trans and North west Himalaya, covers 55,672 km<sup>2</sup> area, 9% of IHR (Figure 1). Like other states of IHR, Uttarakhand has a representative natural and socio-economically important biodiversity. It has a large altitudinal range (200 to 7105 m), with diverse habitats, species, populations, communities and ecosystems. The state embodying Kumaun region (28° 51' N latitude and 30° 49' E longitude) and Garhwal region (77° 33' 5" to 80° 6' E longitude and 29° 31' 9" to 31° 26' 5" N latitude) (Nand and Kumar, 1989), centers of spiritual knowledge, religiosity and pilgrimage from ancient times and it is also rich in biodiversity. The study was conducted in sub-temperate to sub-alpine zones of both Kumaun (Kilbury, Jageshwar, Doonagiri, Chaubattia, Abott mount) and Garhwal (Bhavisya Badri (Chamoli), Tungnath (Rudraprayag), Dayara (Uttarkashi), Bharsar, Binsor (Pauri)), at five different sites to calculate the population status and for preparing conservation strategies for further research.

#### FIELD METHODS

##### Selection, sampling and population estimation

*P. verticillatum* (Linn.) All. and *P. cirrhifolium* (Wall.) Royle, which have high trade and conservation value, were selected for the present study (Plate 1). The population assessment of this species was carried out from May, 2010 to May, 2012, during the peak season of flowering for the study of plant diversity and specimen collection from all the altitudinal zones. The study area was surveyed extensively and 10 populations (5 in Kumaun region and 5 in Garhwal region) mentioned in Table 1 were identified for comparative assessment of the species. Various habitats were





Figure 1. Site map: Uttarakhand.

Table 1. Region-wise selected study sites.

Region	Selected study sites
Kumaun	Kilbury (Nainital District), Jageshyar, Chaubattia, Doonagiri (Almora District), Abott Mount (Champawat District)
Garhwal	Bhavisya Badri (Chamoli District), Tungnath (Rudraprayag District), Dayara (Uttarkashi District), Bharsar, Binsor (Pauri District)

identified based on altitude, topography, presence of perennial water channels and physiognomy of vegetation. Fresh samples of each species were collected and identified with the help of flora (Gaur, 1999; Naithani, 1984), herbarium of Regional Research Institute of Himalayan Flora, Thapla, Ranikhet (Acronym-Rkt) and Taxonomists.

For the phytosociological study in every study sites, herbaceous species was studied by laying 30 quadrats of 1 m × 1 m (1 sq m) size randomly, tree species and shrub species were studied by laying 30 quadrats of 10 m × 10 m (100 sq m) size at different altitudinal range (Misra, 1968). The size and the number of quadrats were determined by the species curve (Mishra, 1968) and the running means methods (Kershaw, 1973). In each quadrat, trees were recorded with > 31.5 cm cbh (circumference at breast height that is, 1.37 m above the ground) individually measured. Individuals within the cbh range of 10.5 to 31.4 cm were considered as shrubs + saplings and individuals < 10.5 cm cbh were considered as herbs + seedlings. Individuals of all species were counted in each quadrat. To determine status of the species, mean values of each quantitative parameter of three stands of transect were considered for further interpretation. During the population analysis, some sites where individuals of representative species were very few (1 to 5) are not considered as stands. However, these individuals were also marked and counted as area of occurrence and for the demographic observations for threat category assessment.

The threat category of *P. verticillatum* (Linn.) All. and *P. cirrhifolium* (Wall.) Royle were identified using six attributes (that is, habitat preference, distribution range, population size, use pattern, extraction trend, native and endemic species) and following Samant et al. (1998) and Ved et al. (2003) (Table 2).

#### Quantitative analysis

The important quantitative analysis such as density, frequency and abundance of tree species, shrubs and herbs species were determined as per Curtis and McIntosh (1950). Pattern of the species was analyzed on the basis of abundance to frequency (A/F) ratio. Value of A/F < 0.025 was categorized regular, between 0.026 to 0.050 random and > 0.050 contiguous type of distribution (Kershaw, 1973). Similarly, relative values of frequency, density and dominance and importance value index (IVI), were calculated following the methods of Curtis (1959). IVI was calculated through the sum of relative frequency, relative density and relative dominance.

#### Statistical analysis

To calculate the significant and non-significant variations among various phytosociological features (frequency, density, abundance



**Table 2.** Threat assessment of the medicinal plant diversity.

Habitat	Distribution	Population (Ind/Location)	Use pattern	Extraction trend	Native and endemic	Score
Single	<500	250 Ind/ upto 2 locality	4 and >4	Commercial	Native and Endemic	10
2-3	500-1000	250–1000 Ind/3–5 locality	2-3	Self use	Native/ Endemic	6
>3	>1000	1000 Ind/>5 locality	Single	No Use	Non Native	2

**Table 3.** Study sites with dominant species at Kumaun.

Code	Location	Latitude/ Longitude	Altitude (m)	Dominant species
P1	Kilburry	29°23'56.36"N/79°26'80"E	2210	<i>Ageratum conizoides</i> L., <i>Anisomeles indica</i> Edgew., <i>Blumea laciniata</i> (Roxb.) DC., <i>Ranunculus arvensis</i> L.
P2	Jageshwar	29°38'59.99"N/79°34'59.98"E	2020	<i>Anaphalis contorta</i> (D.Don) Hook.f., <i>Cynoglossum zeylanicum</i> (Vahl ex Hornem.) Thunb. Ex Lehm., <i>Scutellaria repens</i> Buch.-Ham. ex D.Don, <i>Anemone obtusiloba</i> D.Don
P3	Duangiri	30°30'54"N/79°52'E	2400	<i>Asparagus adscendens</i> Roxb., <i>Ageratum conizoides</i> L., <i>Ajuga bracteosa</i> Wall. ex Benth., <i>Themeda arundinacea</i> (Roxb.) Ridley
P4	Chaubattia	29°36'49.61"N/79°27'22.77"E	1829	<i>Adiantum lunulatum</i> Burm., <i>Boenninghausenia albiflora</i> (Hook.) Meisn., <i>Thalictrum foliolosum</i> DC., <i>Galinsoga parviflora</i> Cav.
P5	Abott Mount	29°24'13.69"N/80°5' 24.43"E	2133	<i>Taraxicum officinale</i> Weber, <i>Paspalum scrobiculatum</i> L., <i>Oxalis corniculata</i> L., <i>Origanum vulgare</i> L.

and IVI), single factor analysis of variance (ANOVA) was applied (Data analysis tool Pack- MS office-2007).

#### Ethno-botanical assessment

To access the ethno-medicinal uses of the plants, personnel interview and bilateral discussion were carried out in the premises of healers/informers. After the documentation of folk claims, validation of the information was also done through cross checking with the help of codified texts of Ayurveda.

## RESULTS

The site and habitat characteristics have been presented in Tables 3 and 4 for Kumaun and Garhwal

region of Uttarakhand, respectively. The area surveyed in Kumaun varying in the altitudinal gradient from 1,829 to 2,210 m while in Garhwal, altitude is ranging from 2,480 to 3,680 m. To access the structural pattern of *Polygonatum verticillatum* (Linn.) All., five sites were accessed in both regions of Uttarakhand namely Kumaun (Kilburry (P1), Jageshwar (P2), Dunagiri (P3), Chaubattia (P4), Abott Mount (P5) and Garhwal (Bhavisya Badri (P6), Tungnath (P7), Dayara (P8), Bharsar (P9), Binsor (P10).

#### Structural pattern of *P. verticillatum* (Linn.) All.

In Kumaun, at site P3 the species had lowest

frequency (50), with the density 1.13 plants/m<sup>2</sup> and IVI of 8.99.

While in Garhwal, at site P9 the species showed lowest frequency (43.33), with the density of 2.27 plants/m<sup>2</sup> and IVI = 15.69 (Tables 5 and 6).

#### Structural pattern of *P. cirrhifolium* Royle

In Kumaun, at site P4 the species had the lowest frequency (50), with a density of 0.87 plants/m<sup>2</sup> and IVI of 8.99. While in Garhwal, at site P7 the species showed lowest frequency (53.33) with the density of 1.70 plants/m<sup>2</sup> and IVI of 15.69 (Tables 7 and 8).

**Table 4.** Study sites with dominant species at Garhwal.

Code	Location	Latitude/Longitude	Altitude (m)	Dominant species
P6	Bhavisya Badri	30°28.738"N/79°40'.604"E	2,744	<i>Ageratum conizoides</i> L., <i>Anisomeles indica</i> Edgew, <i>Blumea laciniata</i> (Roxb.) DC., <i>Asparagus racemosus</i> Willd.
P7	Tungnath	30°34.632"N/79°06'.937"E	3,680	<i>Anaphalis contorta</i> (D.Don) Hook.f., <i>Cynoglossum zeylanicum</i> (Vahl ex Hornem.) Thunb. Ex Lehm., <i>Scutellaria repens</i> Buch.-Ham. ex D.Don, <i>Blumea laciniata</i> (Roxb.) DC.
P8	Dayara	30°83.49"N/78°54'.37"E	3,048	<i>Asparagus adscendens</i> Roxb., <i>Ageratum conizoides</i> L., <i>Ajuga bracteosa</i> Wall. ex Benth., <i>Themeda arundinacea</i> (Roxb.) Ridley
P9	Bharsar	30°06.52"N/78°99'.81"E	2,550	<i>Adiantum lunulatum</i> Burm., <i>Boenninghausenia albiflora</i> (Hook.) Meisn., <i>Thalictrum foliolosum</i> DC., <i>Galinsoga parviflora</i> Cav.
P10	Binsor	29°58.23"N/79°09'.39"E	2,480	<i>Taraxicum officinale</i> Weber, <i>Paspalum scrobiculatum</i> L., <i>Oxalis corniculata</i> L., <i>Origanum vulgare</i> L.

**Table 5.** Phytosociological features of *Polygonatum verticillatum* (Linn.) All. in 5 sites of Kumaun

Site code	Frequency	Density/m <sup>2</sup>	Abundance	IVI
1	53.33±0.37	1.20±0.12	2.25±0.15	12.02±0.14
2	66.67±1.82	1.73±0.32	2.60±0.42	12.06±0.26
3	50.00±0.26	1.13±0.21	2.27±0.024	8.99±0.65
4	70.00±2.15	1.27±0.25	1.81±0.28	11.60±0.37
5	60.00±3.32	1.63±0.24	2.72±0.27	13.75±0.82
F value significant at P = 0.05	1.91 <sup>NS</sup>	17.17***	42.07***	38.56***

**Table 6.** Phytosociological features of *Polygonatum verticillatum* (Linn.) All. in 5 sites of Garhwal.

Site code	Frequency	Density/m <sup>2</sup>	Abundance	IVI
6	70±8.16	2.43±0.34	3.05±0.42	23.65±7.10
7	70±8.16	2.83±0.29	3.54±0.36	24.14±6.57
8	60±8.16	2.60±0.51	3.71±0.73	21.85±7.46
9	43.33±4.71	2.27±0.29	4.53±0.57	15.69±2.51
10	60±8.16	22.95±0.18	4.42±0.20	51.19±13.01
F value significant at P = 0.05	1.38 <sup>NS</sup>	0.83 <sup>NS</sup>	0.83 <sup>NS</sup>	0.01*

**Table 7.** Phytosociological features of *Polygonatum cirrhifolium* Royle in 5 sites of Kumaun.

Site Code	Frequency	Density/m <sup>2</sup>	Abundance	IVI
1	70.00±2.17	1.13±0.11	1.62±0.07	13.38±0.13
2	50.00±2.16	1.60±0.12	3.20±0.10	14.06±0.21
3	56.67±1.86	0.63±0.22	1.12±0.06	9.63±0.17
4	50.00±2.31	0.87±0.20	1.53±0.09	11.39±0.21
5	76.67±3.26	1.90±0.55	2.48±0.36	11.01±0.19
F value significant at P = 0.05	6.00**	28.07***	58.61***	187.86***

**Table 8.** Phytosociological features of *Polygonatum cirrhifolium* Royle in 5 sites of Garhwal.

Site code	Frequency	Density/m <sup>2</sup>	Abundance	IVI
6	56.67±4.71	1.23±0.21	2.06±0.34	10.45±1.48
7	53.33±4.71	1.70±0.64	3.40±0.75	13.73±1.97
8	60±8.16	2.27±0.05	3.77±0.07	26.93±6.64
9	70±8.16	2.87±0.90	4.10±1.28	28.85±5.82
10	60±8.16	3.20±0.51	4.57±0.73	28.62±4.79
F value significant at P = 0.05	1.59 <sup>NS</sup>	4.31*	3.18 <sup>NS</sup>	7.31**

### Statistical analysis

For *P. verticillatum*, in Kumaun, frequency (F = 1.91, P = 0.05, df = 14) was found non-significant while density (F = 17.17, P = 0.05, df = 14), abundance (F = 42.07, P = 0.05, df = 14) and IVI (F = 38.56, P = 0.05, df = 14) were found highly significant. In Garhwal, frequency (F = 1.38, P = 0.05, df = 14), density (F = 0.83, P = 0.05, df = 14) and abundance (F = 0.83, P = 0.05, df = 14) of the plant species were found non-significant, while IVI (F = 0.01, p = 0.05, df = 14) value was found significant. In Kumaun, frequency of *P. cirrhifolium* was found highly significant (F = 6.00, P = 0.05, df = 14). Density (F = 28.07, P = 0.05, df = 14), abundance (F = 58.61, P = 0.05, df = 14) and IVI (F = 187.86, P = 0.05, df = 14) were also found highly significant. In Kumaun, frequency (F = 1.59, P = 0.05, df = 14) and abundance (F = 3.18, P = 0.05, df = 14) were found non-significant while density (F = 4.31, P = 0.05, df = 14) and IVI (F = 7.31, P = 0.05, df = 14) were found significant.

### Threat categorization

An area specific threat categorization is very important for short or long term management planning. In the present study, the threat categorization of the species was done through the six parameters (that is, habitat preference, distribution range, population size, use pattern, extraction trend, native and endemic species). Both the species *P. verticillatum* (Linn.) All. and *P. cirrhifolium* Royle are found vulnerable in the study area on the basis of six parameters stated earlier. This indicates that these species

are facing threats due to high anthropogenic pressure, overexploitation, habitat destruction and fragmentation.

### Ethno-botanical uses

*P. verticillatum* (Linn.) All. and *P. cirrhifolium* Royle are the main ingredients of Astaverga (an Ayurvedic formulation). As per Ayurveda (Sharma et al., 1979), the main property of these two plants is to treat the vata, pitta, general weakness, aphrodisiac etc. During the field survey, some ethno-medicinal uses of *P. verticillatum* (Linn.) All. and *P. cirrhifolium* Royle were also accessed, which are more or less same for both plants.

1. Bulbs of *P. verticillatum* (Linn.) All. are dried, powdered and taken after mixing with honey to cure tuberculosis.
2. Powder of bulbs is taken with milk to cure general debility, and acts as tonic.
3. Powder of bulbs is taken twice daily with water to cure leucorrhoea.

### DISCUSSION

Both the species of *Polygonatum* are used variously by pharmaceutical companies, due to which the population in natural habitat is diminishing day by day at alarming stage. The present study is the population assessment of the species that is, *P. verticillatum* (Linn.) All. and *P. cirrhifolium* Royle following the parameters given by the Mishra (1968), to define the status of the plant in the study area and to access the ethno-medicinal uses of the

plant. The study reveals that both the species are vulnerable in the study area and facing the various kind of threats such as: over exploitation, unscientific harvesting and less awareness about the properties of the species. There are some strategies for the conservation of these species for future prospects, such as:

1. A thorough work on threatened medicinal plants should be carried out and detailed information about their natural habitat, climate, soil, adaptability, growing season, flowering time, seed setting stage, etc., should be generated. Accordingly, conservation measures should be developed.
2. Ban on threatened medicinal plants should be strictly materialized, since due to improper attention of administration one can easily see banned medicinal plants in the market.
3. Collection of folklore information on medicinal plants from tribal and elderly people and its proper documentation are very important, otherwise this valuable information will vanish with them and coming generation will be deprived of it.

## ACKNOWLEDGEMENT

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Full Length Research Paper

## Study on callus induction and plant regeneration of *Leuzea carthamoides* via tissue culture system

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*Leuzea (Rhaponticum carthamoides)* is a valuable medicinal plant from Asteraceae. Micropropagation could be a good alternative for the mass propagation of *Leuzea carthamoides*. To investigate the callogenesis of leaf explants, 12 different hormonal combinations including different concentrations of 16-benzylaminopurine (BA) and 2,4-dichlorophenoxyacetic acid (2, 4-D) were studied in two separable experiments. In both experiments, the explants were transferred to the Ms medium supplemented with 0.5 mg L<sup>-1</sup> indole acetic acid (IAA) and 0.5 mg L<sup>-1</sup> BA for 7 and 50 days after culture for regeneration, respectively. Then, after one month the percentages of callogenesis and the amount of produced callus were measured. In other experiment to investigated regeneration of root explants, 9 different hormonal combinations were studied including different concentrations of BA and IAA. The number of leaf per explants, length of greatest leaf per explant and regeneration percentage were measured one month after culture. The maximum callus production was obtained using 1 mg L<sup>-1</sup> 2, 4-D and 1.5 mg L<sup>-1</sup> BA and 0.25 mg L<sup>-1</sup> 2, 4-D and 1.5 mg L<sup>-1</sup> BA in first experiment and second experiment, respectively. In the third experiment, root explants had direct regeneration and medium with 0.5 mg L<sup>-1</sup> BA + 1 mg L<sup>-1</sup> IAA can be suitable medium.

**Key words:** *Leuzea carthamoides*, *in vitro*, regeneration, callus induction.

### INTRODUCTION

*Leuzea (Rhaponticum carthamoides)* is a valuable medicinal plant from the family Asteraceae (Orlova et al., 2000). *R. carthamoides* is a perennial herb, commonly known as a maral root or Russian leuzea, which has been used for centuries in Eastern parts of Russia due to its marked medicinal properties (Kokoska and Janovska, 2009). The West and East of Siberia, Northern Mongolia and central Asia are its natural habitats. It is a medicinal herb with a tonic effect (Selepcova et al., 1993). Several different classes of compounds were previously isolated from various parts of *R. carthamoides* of which the main groups are steroids, particularly ecdysteroids, and phenolics

phenolics (flavonoides and phenolic acids) accompanied with polyacetylenes, sesquiterpene lactones, triterpenoid glycosides and terpenes (essential oil) (Kokoska and Janovska, 2009). 20-hydroxy-Ecdison or Leuzine is the most important compound present in ecdysterone (Omidbaigi, 2007). This plant is a hidden jewel. *R. carthamoides* extract (RCE) has demonstrated a normalizing effect on central nervous and cardiovascular systems. RCE improves sleep, appetite, moods, mental and physical state, and functional ability of humans under working conditions (Yance, 2004).

*In vitro* cell and tissue culture methodology is envisaged

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as a means for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale revegetation, and for genetic manipulation studies (Nalawade et al., 2003).

The conducted researches on micropropagation of *Leuzea carthamoides* are inadequate. Orlova et al. (2000) used MS medium supplemented with 1 mg L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2, 4-D) and 1 mg L<sup>-1</sup> 16-benzylaminopurine (BA) for Callus induction. They also used Murashige and Skoog (MS) medium supplemented with 0.5 mg L<sup>-1</sup> Indole acetic acid (IAA) and 0.5 mg L<sup>-1</sup> BA for regeneration and MS medium with the addition of 0.5 mg L<sup>-1</sup> IBA and 0.2 mg L<sup>-1</sup> BA for shoot propagation. Duskova and Dusek (1995) reported that Calluses derived from the aerial parts grew best on MS media supplemented with 1.0 mg 2, 4-D + 0.5 mg IBA/liter (544% increase in FW after 4 weeks). Callus cultures derived from the roots grew less well; the best results were obtained with 1.0 mg 2, 4-D + 1.0 mg BA/liter (230%). They also said that bud formation occurred on partly callused cotyledonary leaflets on media supplemented with 1.0 mg IAA/liter. Akhmetova and Baiburina (2002) reported that in case of micropropagation of *Leuzea carthamoides*, the best results were obtained using the receptacles of young heads of *R. carthamoides* as an explant, and 0.2 mg IBA + 0.2 mg NAA/liter, or 0.5 mg IBA/liter.

The present study aims to determine the effect of different concentrations of (BA) and (2,4-D) on callus induction of *L. carthamoides* through the culture of leaf explants and investigate the effect of different concentrations of BA and IAA on regeneration of root explants.

## MATERIALS AND METHODS

Leaf explants of *L. carthamoides* were obtained from Zardband research garden (elevation 1548 m above sea level, latitude 3547 North of Tehran) in June, 2008. The leaf explants were transferred to the laboratory after collection. Initially, the explants were washed under running tap water for 30 min to 1 h and then leaves were divided into small pieces and surface-sterilized by immersion in ethanol (70% v/v) for ten seconds continued by 1% (w/v) sodium hypochlorite solution for 18 min. Afterward, the plant materials were rinsed in sterile distilled water three times and finally, the leaf explants were prepared. The medium consisted MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar that was supplemented with different hormonal combinations in each experiment. The pH of the medium was regulated to 5.7 and autoclaved at 121°C for 20 min. All cultures were incubated at 25 ± 2°C under a 16 h photoperiod provided by cool white fluorescent tubes.

In this study, to investigate callus induction from leaf explant 12 different hormonal combinations including different concentrations of BA and 2,4-D were studied in two distinguishable experiments (Table 2). These experiments were conducted in factorial based on a completely randomized design (CRD) with two factors and three replications. BA at three levels and 2, 4-D at four levels were used;

each replication consisted of one petri-dish (with 10 cm diameter) with three leaf explants. In first experiment after one week, the explants were transferred to the MS medium supplemented with 0.5 mg L<sup>-1</sup> IAA and 0.5 mg L<sup>-1</sup> BA for regeneration. After one month, the percentages of callogenesis (number of callogenic explants / total number of explants × 100), the rates of explants callogenesis and the percentages of regeneration (number of regenerated explants / total number of explants × 100) were measured.

In second experiment after 20 days, the explants were transferred to the same medium for one additional month. Then explants were transferred to the MS medium supplemented with 0.5 mg L<sup>-1</sup> IAA and 0.5 mg L<sup>-1</sup> BA for regeneration. After one month, the percentages of callogenesis, the rates of explants callogenesis were measured. The rates of explants callogenesis were identified with codes (Code 0: explants producing no callus or gone black; Code 1: explants producing little callus (< 50 mm<sup>2</sup>); Code 2: explants producing a little callus (50 to 100 mm<sup>2</sup>); Code 3: explants producing average amount of callus (100 to 200 mm<sup>2</sup>); Code 4: explants producing much callus (200 to 300 mm<sup>2</sup>); and Code 5: explants producing too much callus (> 300). To shoot propagation, plantlets obtained from first experiment were transferred to MS medium supplemented with 0.5 mg L<sup>-1</sup> IBA and 0.2 mg L<sup>-1</sup> BA. Finally, MS medium without hormone was used for root production of plantlets.

In third experiment, root explants were taken from these plantlets. In this experiment, to study regeneration of root explants, 9 different hormonal combinations were applied including different concentrations of BA and IAA (Table 6). This experiment was carried out in factorial based on a completely randomized design (CRD) with two factors and four replications. Each replication consisted of one Petri dish (with 6 cm diameter) with three leaf explants. The number of leaf per explants, length of greatest leaf per explant and regeneration percent were measured one month after culture. For acclimatization, the rooted plantlets were taken out and tenderly washed in tap water to remove all traces of the media. Subsequently, they were planted in plastic cups (upper diameter 7.5 cm × length 8 cm, with a volume of 240 cm<sup>3</sup>) filled with peat moss purchased from Slovenia. Then they were kept in greenhouse at 29°C in day and 25°C in night and relative moisture more than 90% at the first 14 days and 70% at the next 30 days.

Data were analyzed by one-way analysis of variance (ANOVA) and the means were evaluated using Duncan's new multiple range test (DMRT) at the 5% level. In related traits such as explants weight, leaf number, length of greatest leaf, and plantlet height data analysis was carried out using SAS Version 9.1 (SAS Institute, 2002). Ranking data were analyzed by Kruskal Wallis nonparametric test.

## RESULTS

About 98.6 and 94.44% of explants produced callus in the first experiment and second experiment, respectively. However, the results (Table 1) showed that treatments differed significantly in callus surface. In first experiment, the concentration of 1 mg L<sup>-1</sup> 2, 4-D and 1.5 mg L<sup>-1</sup> BA resulted in the highest callus surface. The concentration of 0.25 mg L<sup>-1</sup> 2, 4-D and 1.5 mg L<sup>-1</sup> BA resulted in the most callus surface in second experiment (Table 2). In first experiment, Code 1 (explants producing little callus) had the maximum percentages among other codes.

Whereas in second experiment code 3 (explants

**Table 1.** Data analysis of the produced callus surface in leaf explants in first experiment and second experiment.

Source of variation	Degree of freedom	Chi-Square of callus surface in first experiment	Chi-Square of callus surface in second experiment
Treatment	11	19.53*	45.06**

\*, \*\*significant at 0.05 and 0.01 level, respectively.

**Table 2.** Average rank of the produced callus surface in leaf explants.

Treatment (2,4-D+BA) (mg L <sup>-1</sup> )	Average rank in first experiment	Average rank in second experiment
H <sub>1</sub> (1 + 1.5)	3.85	2.5
H <sub>2</sub> (1 + 1)	2.85	3.8
H <sub>3</sub> (1 + 0.5)	1.57	2.8
H <sub>4</sub> (0.75 + 1.5)	2.85	3.5
H <sub>5</sub> (0.75 + 1)	2.28	3.6
H <sub>6</sub> (0.75 + 0.5)	2	3.6
H <sub>7</sub> (0.5 + 1.5)	1	2.6
H <sub>8</sub> (0.5 + 1)	1.57	3.8
H <sub>9</sub> (0.5 + 0.5)	1.42	3.3
H <sub>10</sub> (0.25 + 1.5)	3.42	4.1
H <sub>11</sub> (0.25 + 1)	1.28	2.6
H <sub>12</sub> (0.25 + 0.5)	2.85	2.5

**Table 3.** Results of data analysis of the produced callus surface in different times (incubate in medium consisted 2, 4-D).

Source of variation	df	Mean square
Time	1	14.93**

\*\*significant at 0.01 level

**Table 4.** The results of ANOVA for the effects of 2,4-D and BA on regeneration of leaf explants of *Leuzea carthamoides* in first experiment.

Source of variation	df	Mean square
2,4-D (A)	3	2551.48**
BA (B)	2	401.22 <sup>ns</sup>
A×B	6	1718.09**
Experimental error	24	370.35

\*, \*\*significant at 0.05 and 0.01 level, respectively; Ns: not significant.

producing average amount of callus) and thereafter code 4 (explants producing much callus) had the maximum percentages among other codes (Figure 1). Based on

Table 3, there was significant difference between amounts of callus produced in term of time. In the first experiment, rapid transmission into medium containing caused the explants regenerated rapidly, whereas in second experiment, with longer culturing of explants on medium supplemented with 2, 4-D and BA produced more callus amount and regenerated later than explants in first experiment. Figures 2 and 3 show Callogenesis and regeneration of leaf explants in first experiment and second experiment, respectively.

Regeneration was obtained from leaf explants in first experiment within 18 to 21 days after transfer into medium containing 0.5 mg L<sup>-1</sup> IAA and 0.5 mg L<sup>-1</sup> BA. Therefore, in the first experiment, regeneration percent were measured simultaneous with measurement of the rates of explants callogenesis. The effects of 2, 4-D and BA on regeneration of leaf explants was statistically significant (Table 4). Based on result, the maximum regeneration percent was acquired by using 0.25 mg L<sup>-1</sup> 2, 4-D and 1.5 mg L<sup>-1</sup> BA (Table 5). Results obtained from the first and second experiment were in agreement with the findings of Orlova et al. (2000). It was established that using of medium supplemented with 2,4-D and BA and rapidly transfer from this medium, the efficiency of plant regeneration from leaf explants increased and regeneration occurred rapidly and with longer culturing on this medium produced callus tissues incapable of regenerating



**Table 5.** Effect of different concentration of 2,4-D and BA on regeneration of leaf explants in first experiment.

Treatment (2,4-D+BA) (mg L <sup>-1</sup> )	Shoot regeneration (%)
H <sub>1</sub> (1 + 1.5)	44.44 <sup>bc</sup>
H <sub>2</sub> (1 + 1)	11.11 <sup>dc</sup>
H <sub>3</sub> (1 + 0.5)	55.56 <sup>ab</sup>
H <sub>4</sub> (0.75 + 1.5)	66.67 <sup>ab</sup>
H <sub>5</sub> (0.75 + 1)	44.44 <sup>bc</sup>
H <sub>6</sub> (0.75 + 0.5)	44.44 <sup>bc</sup>
H <sub>7</sub> (0.5 + 1.5)	0 <sup>d</sup>
H <sub>8</sub> (0.5 + 1)	55.56 <sup>ab</sup>
H <sub>9</sub> (0.5 + 0.5)	44.44 <sup>bc</sup>
H <sub>10</sub> (0.25 + 1.5)	88.89 <sup>a</sup>
H <sub>11</sub> (0.25 + 1)	55.56 <sup>ab</sup>
H <sub>12</sub> (0.25 + 0.5)	66.67 <sup>ab</sup>

Means in columns with different letters are significantly different at ( $P \leq 0.05$ ).

**Table 6.** Effect of different concentration of IAA and BA on regeneration percent of root explants and Length of greatest leaf of plantlets produced from root explants.

Treatment (BA+IAA) (mg L <sup>-1</sup> )	Mean $\pm$ SE	
	Regeneration percent	Length of greatest leaf (cm)
1 (1.5 + 1.5)	25 $\pm$ 25 <sup>b</sup>	0.63 $\pm$ 0.63 <sup>c</sup>
2(1.5 + 1)	85.42 $\pm$ 8.58 <sup>a</sup>	1.93 $\pm$ 0.08 <sup>ab</sup>
3 (1.5 + 0.5)	91.67 $\pm$ 8.32 <sup>a</sup>	1.01 $\pm$ 0.14 <sup>bc</sup>
4 (1 + 1.5)	100 $\pm$ 0 <sup>a</sup>	1.57 $\pm$ 0.23 <sup>abc</sup>
5 (1 + 1)	75 $\pm$ 25 <sup>a</sup>	0.57 $\pm$ 0.09 <sup>c</sup>
6 (1 + 0.5)	100 $\pm$ 0 <sup>a</sup>	1.38 $\pm$ 0.08 <sup>abc</sup>
7 (0.5 + 1.5)	91.67 $\pm$ 8.32 <sup>a</sup>	1.37 $\pm$ 0.25 <sup>abc</sup>
8 (0.5 + 1)	100 $\pm$ 0 <sup>a</sup>	2.32 $\pm$ 0.26 <sup>a</sup>
9 (0.5 + 0.5)	91.67 $\pm$ 8.32 <sup>a</sup>	0.96 $\pm$ 0.31b <sup>c</sup>

Means in columns with different letters are significantly different at ( $P \leq 0.05$ ).

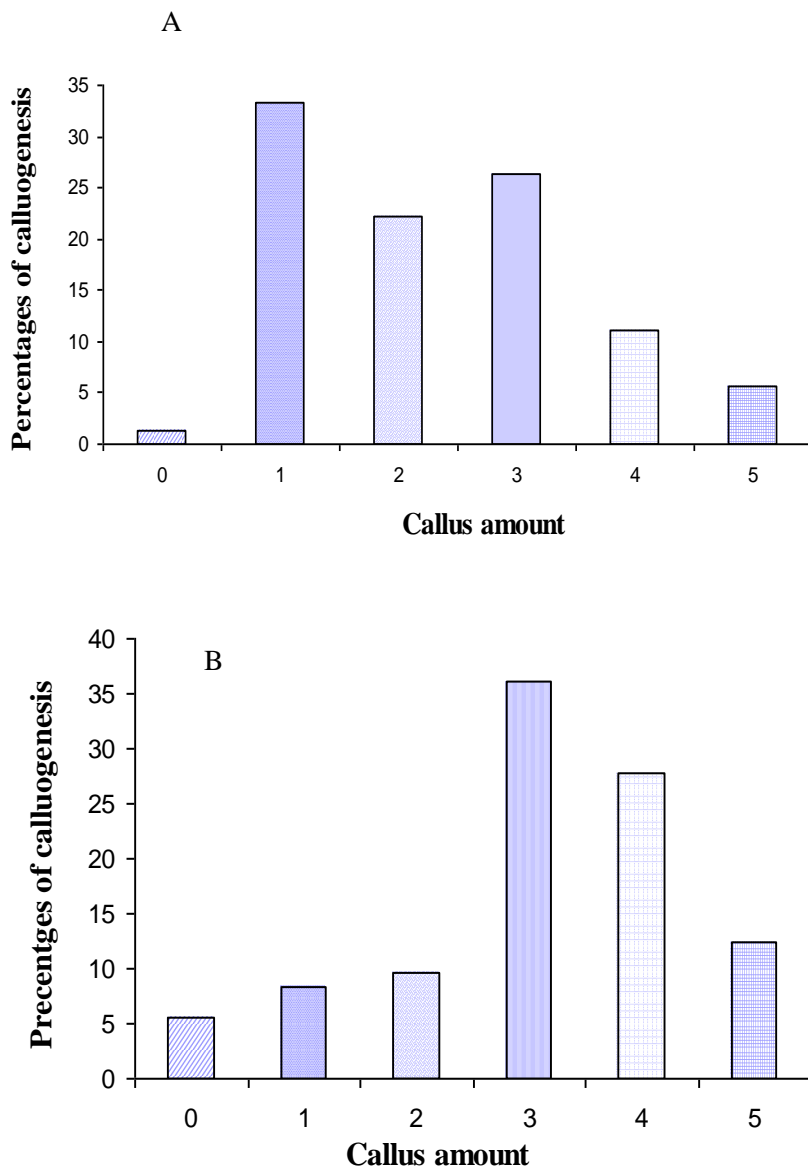
plants. Shoot propagation of plantlets made on MS medium supplemented with 0.5 mg L<sup>-1</sup> IBA and 0.2 mg L<sup>-1</sup> BA. Orlova et al. (2000) used the basal medium A contained MS salts, vitamins according to Staba, 8 g L<sup>-1</sup> agar or agarose, and 30 g L<sup>-1</sup> sucrose supplemented with 0.5 mg L<sup>-1</sup> IBA and 0.2 mg L<sup>-1</sup> BA for the shoot propagation. The rooted plantlets were optimized within one month, and then were planted in Zardband research garden (Figure 5).

In the the third experiment, root explants had direct regeneration (Figure 4). The effect of hormone was statistically significant in the case of the regeneration percent and length of greatest leaf per explants, but the hormone treatment did not have any statistically significant effect on the number of leaf produced per explants (Table 6). The medium containing 1 mg L<sup>-1</sup> BA +

**Table 7.** Main effect of BA on leaf number.

BA (mg l <sup>-1</sup> )	Leaf number
1.5	18.94 <sup>b</sup>
1	28.55 <sup>ab</sup>
0.5	35.29 <sup>a</sup>

1.5 mg L<sup>-1</sup> IAA, 1 mg L<sup>-1</sup> BA + 0.5 mg L<sup>-1</sup> IAA and 0.5 mg L<sup>-1</sup> BA + 1 mg L<sup>-1</sup> IAA, produced the maximum regeneration percent (100%). Longest leaf (2.32 cm) was obtained when 0.5 mg L<sup>-1</sup> BA + 1 mg L<sup>-1</sup> IAA was employed (Table 6). Therefore, MS medium with 0.5 mg L<sup>-1</sup> BA + 1 mg L<sup>-1</sup> IAA can be a suitable medium for direct regeneration of root explants. Table 7 shows main effect



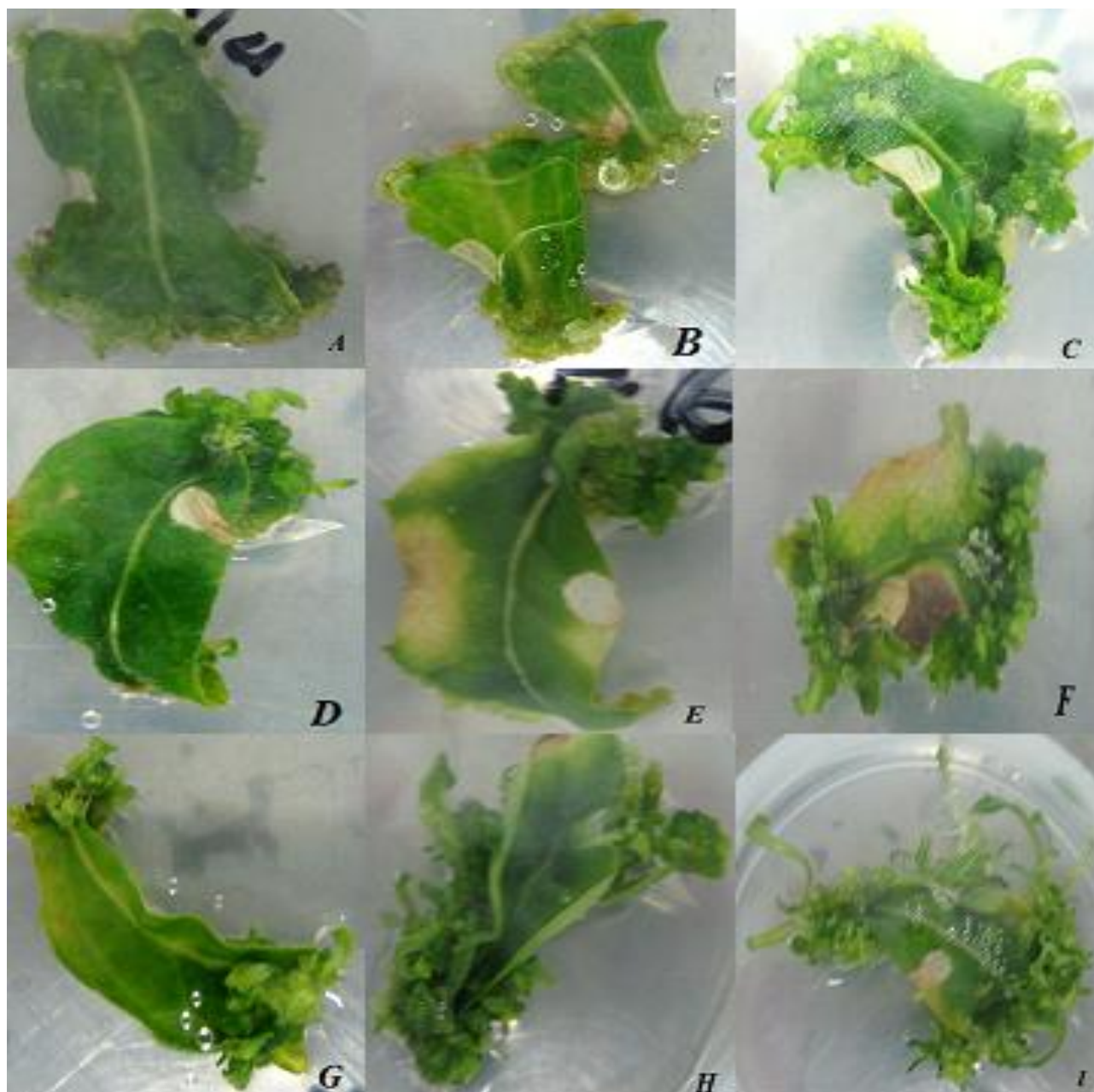
**Figure 1.** Study of rates of leaf explants calluogenesis. A: in first experiment, B: in second experiment.  
 (Code 0: explants producing no calli or gone black; Code 1: explants producing little callus (< 50 mm<sup>2</sup>); Code 2: explants producing a little callus (50 to 100 mm<sup>2</sup>); Code 3: explants producing average amount of callus (100 to 200 mm<sup>2</sup>); Code 4: explants producing much callus (200 to 300 mm<sup>2</sup>); and Code 5: explants producing too much callus (300< ).

of BA on leaf number. The concentration of 0.5 mg L<sup>-1</sup> BA resulted in the most leaf number (35.29).

**Conclusion**

In summary, present experiment showed that use of leaf

and root explants for micropropagation is beneficial. It also can be useful in conservation and genetic transformation studies aimed at improving this plant. In *L. carthamoides*, a combination of BA and 2,4-D was found to be suitable for induction of callus. However, the yield of shoot regeneration was satisfactory. Regeneration was obtained from leaf explants in MS medium containing 0.5

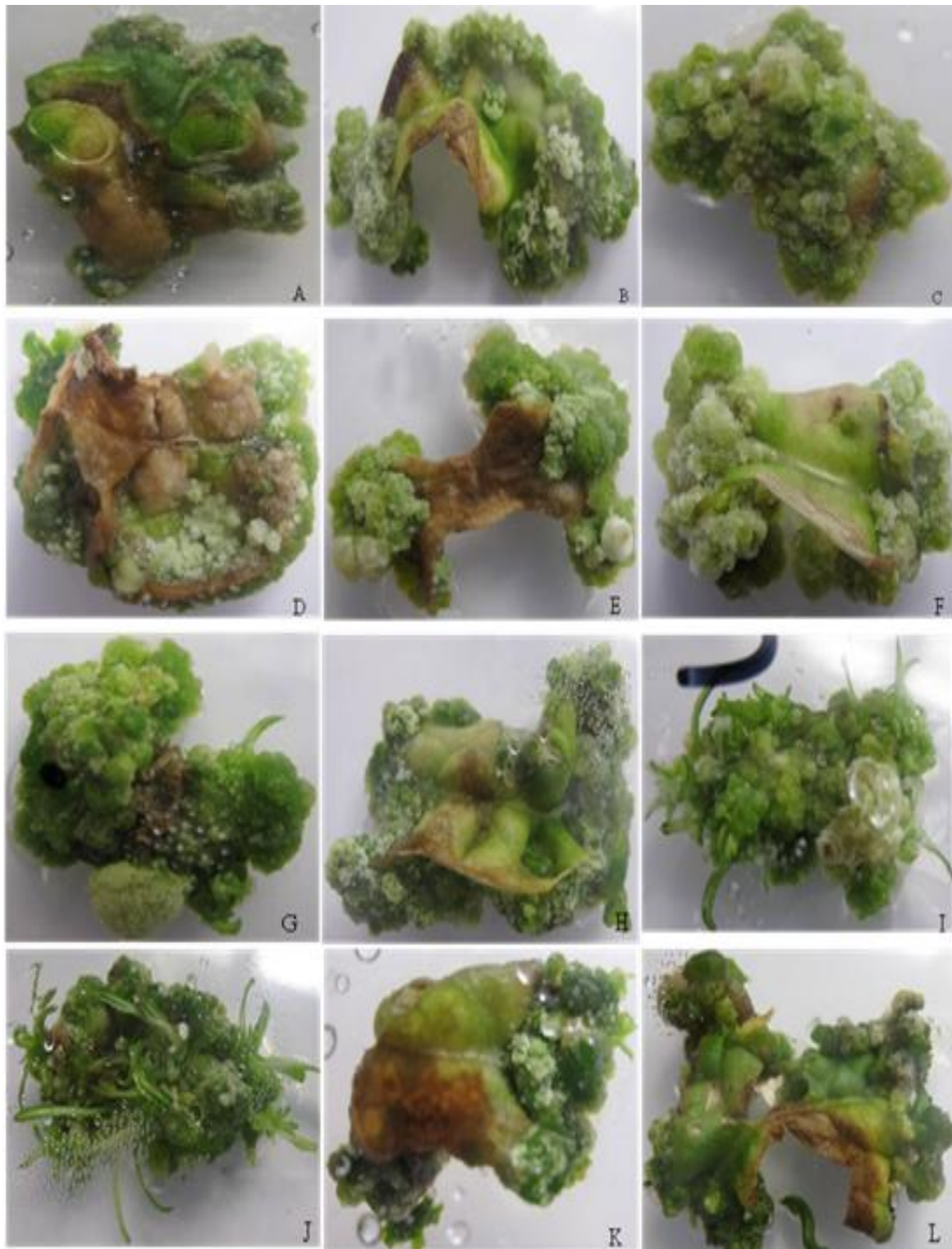


**Figure 2.** Callogenesis and regeneration of leaf explants in first experiment.

A) 2,4-D: 1 mg/L, BA: 1/5 mg/L; B) 2,4-D: 1 mg/L, BA: 1 mg/L; C) 2,4-D: 0/75 mg/L, BA: 1/5 mg/L; D) 2,4-D: 0/75 mg/L, BA: 1 mg/L; E) 2,4-D: 0/75 mg/L, BA: 0/5 mg/L; F) 2,4-D: 0/5 mg/L, BA: 1 mg/L; G) 2,4-D: 0/5 mg/L, BA: 0/5 mg/L; H) 2,4-D: 0/25 mg/L, BA: 1/5 mg/L; I) 2,4-D: 0/25 mg/L, BA: 0/5 mg/L.

mg L<sup>-1</sup> IAA and 0.5 mg L<sup>-1</sup> BA. Orlova et al. (2000) used from MS medium with the addition of 0.5 mg L<sup>-1</sup> IAA and 0.5 mg L<sup>-1</sup> BA for regeneration the plant. The present callus regeneration system may also be important for advanced studies on genetic improvement and in the future, also has considerable potential as an alternative

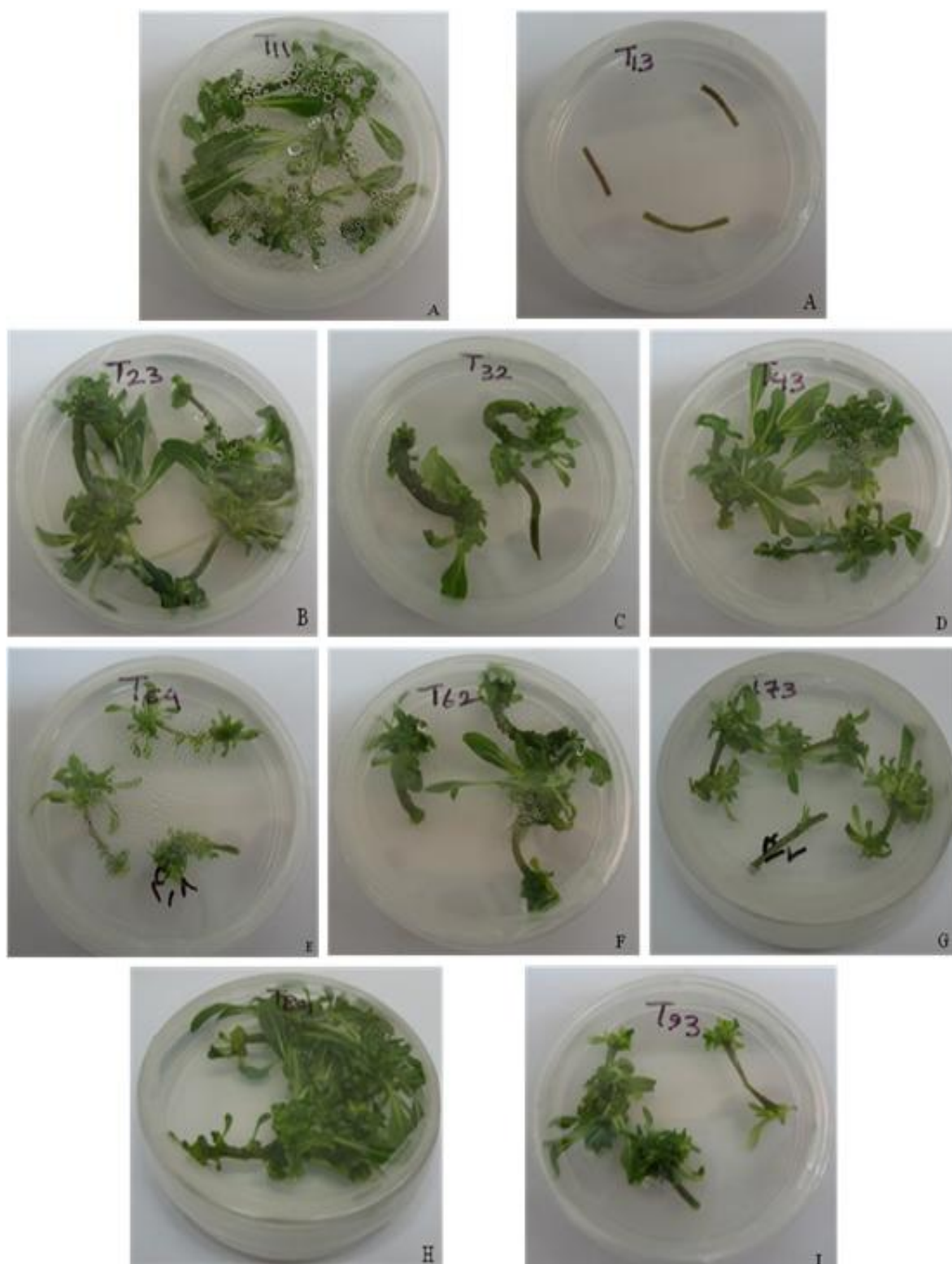
means for production of known and new secondary metabolites. In the third experiment, root explants had direct regeneration. MS medium with 0.5 mg L<sup>-1</sup> BA + 1 mg L<sup>-1</sup> IAA can be suitable medium for direct regeneration of root explants. Regeneration of plantlet from underground stem and leaf explants of *Curculigo orchoides*



**Figure 3.** Callogenesis of leaf explants in second experiment.

A) 2,4-D: 1 mg/L, BA: 1/5 mg/L; B) 2,4-D: 1 mg/L, BA: 1 mg/L; C) 2,4-D: 1 mg/L, BA: 0/5 mg/L; D) 2,4-D: 0/75 mg/L, BA: 1/5 mg/L; E) 2,4-D: 0/75 mg/L, BA: 1 mg/L; F) 2,4-D: 0/75 mg/L, BA: 0/5 mg/L; G) 2,4-D: 0/5 mg/L, BA: 1/5 mg/L; H) 2,4-D: 0/5 mg/L, BA: 1 mg/L; I) 2,4-D: 0/5 mg/L, BA: 0/5 mg/L; J) 2,4-D: 0/25 mg/L, BA: 1/5 mg/L; K) 2,4-D: 0/25 mg/L, BA: 1 mg/L; L) 2,4-D: 0/25 mg/L, BA: 0/5 mg/L.





**Figure 4.** Direct regeneration from root explants of *Leuzea carthamoides* on different hormonal combinations. A) BA: 1.5 mg/L, IAA: 1.5 mg/L; B) BA: 1.5 mg/L, IAA: 1 mg/L; C) BA: 1.5 mg/L, IAA: 0.5 mg/L; D) BA: 1 mg/L, IAA: 1.5 mg/L; E) BA: 1 mg/L, IAA: 1 mg/L; F) BA: 1 mg/L, IAA: 0.5 mg/L; G) BA: 0.5 mg/L, IAA: 1.5 mg/L; H) BA: 0.5 mg/L, IAA: 1 mg/L; I) BA: 0.5 mg/L, IAA: 0.5 mg/L.



**Figure 5.** Hardened plant and transferring to the field.

without intervening callus can be efficiently used to preserve true-to-type traits in the propagules and direct regeneration of plantlets from the explant facilitates rapid multiplication (Suri et al., 1999).

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Full Length Research Paper

# A sensitive high performance liquid chromatography-ultraviolet (HPLC-UV) method for the quantitation of $\alpha$ -obscurine in rat plasma and its application to pharmacokinetic studies

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A simple and sensitive high performance liquid chromatography (HPLC) method for the determination of  $\alpha$ -obscurine in rat plasma has been developed and validated. Separation was achieved on Century Sil EPS C<sub>18</sub> (5  $\mu$ m, 200  $\times$  4.6 mm i.d). The mobile phase, water:methanol:triethanol amine (39:61:0.05 v/v/v), was delivered at a flow rate of 1.0 ml/min. The detector was set at 250 nm. No interfering chromatogram peaks in rat blank plasma was observed. The relationship between  $\alpha$ -obscurine concentration and peak area ratio of  $\alpha$ -obscurine to the internal standard (IS) was linear over the range of 0.354 to 14.16  $\mu$ g/ml. The intra- and inter-day coefficients of variation were  $\leq 2.6$  and  $\leq 3.7\%$ , respectively. The methods of recovery of  $\alpha$ -obscurine was 96.03 to 101.6%, and the extraction recovery was 70.81 to 75.27%.  $\alpha$ -Obscurine was found to be for at least 24 h at retention time (RT) and 2 weeks at -20°C. The method was applied to a pharmacokinetic study of  $\alpha$ -obscurine in rats. This study establishes and validates a determination of rats plasma puerarin in high performance liquid chromatography and.

**Key words:** *Lycopodium japonicum* Thunb, high performance liquid chromatography-ultraviolet (HPLC-UV),  $\alpha$ -obscurine, pharmacokinetics.

## INTRODUCTION

*Lycopodium japonicum* Thunb. has definite treating rheumatism bi-complex pain therapeutic effects (Zheng et al., 2006), and has been widely used in clinical. Recent researches indicates that different doses of *Lycopodium* ethanol extracts can exert an inhibitory effect on secondary reaction of Freund's adjuvant arthritis of rats and a obviously decreased level of serum cytokines (interlukin [IL], tumor necrosis factor-alpha [TNF- $\alpha$ ] and IL-6) (Yin et al., 2008; Wang et al., 2004; Wu and Gu, 2006). It was believed that ethanol extracts of *Lycopodium* were the effective pharmacological composition.  $\alpha$ -Obscurine (Figure 1) was the main component in ethanol extracts, it had the obvious analgesic action in our studies. It is one of *Lycopodium* alkaloids. The analysis about  $\alpha$ -obscurine content determination methods

determination methods has not been reported.

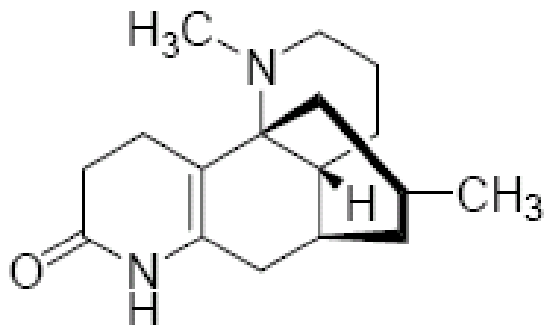
A sensitive and simple analysis method for determining  $\alpha$ -obscurine in rat plasma was established and its pharmacokinetic in rats was studied, because the pharmacokinetic studies on the active ingredients in traditional Chinese medicinal preparations (TCMPs) will contribute to the elucidation of their mechanisms of action.

## EXPERIMENTAL

### Materials, reagents, and solutions

*L. japonicum* Thunb. was obtained from Huaping National Nature Reserve (Guangxi, China).  $\alpha$ -Obscurine was prepared from the





**Figure 1.** Chemical structures of  $\alpha$ -obscurine.

herb of *L. japonicum* Thunb. (purity > 98.5%, determined by high performance liquid chromatography [HPLC]). Schizandrin, used as IS was purchased from the National Institute for Control of Biological and Pharmaceutical Products (Beijing, China).

HPLC-grade methanol was supplied by Yuwang Reagent Co. Ltd. (Shandong, China). Other reagents were of analytical grade. Water was obtained by double distillation. The content of  $\alpha$ -obscurine in the ethanol extract of *Lycopodium* was 78 mg/g.

By dissolving different accurately weighed amounts of standards in methanol solution, the stock solutions were gained as follows:  $\alpha$ -obscurine 141.6  $\mu\text{g/ml}$  and the IS 5  $\mu\text{g/ml}$ . Different volumes of each stock solution were transferred into volumetric flasks and then diluted to scale to make working standard solutions with methanol. All these solutions earlier were stored at 4°C.

### Sample preparation

All plasma samples, quality control samples (QC) samples, and spiked plasma calibration samples were treated in the same manner. To 200  $\mu\text{l}$  plasma, 50  $\mu\text{l}$  IS solution and 1000  $\mu\text{l}$  ether was added. Each tube was mixed thoroughly by vortex mixing for 3 min and then centrifugated at 4000 rpm (800 $\times$ g) for 10 min. The organic phase was transferred into a test tube and evaporated to dryness at 45°C under a gentle stream of nitrogen. Subsequently, the residual was redissolved in 50  $\mu\text{l}$  of methanol and vortexed for 1 min, then centrifugated at 10000 rpm (5500 $\times$ g) for 5 min. Finally, 20  $\mu\text{l}$  of the supernatant was directly injected into the HPLC system.

### Instrumentation and chromatographic conditions

The chromatographic system consisted of an LC-10Atvp liquid chromatograph, and an SPD-10Avp ultraviolet (UV) detector set at 250 nm, all from Shimadzu (Japan). Separation was performed using a prepacked stainless-steel column (200  $\times$  4.6 mm i.d) with Century Sil C<sub>18</sub> EPS 5  $\mu\text{m}$  silica. The mobile phase consisted of 390 ml water, 610 ml methanol, 0.5 ml triethylamine and the flow-rate was 1.0 ml/min.

### Validation of the method

The specificity, linearity, lower limit of quantitation (LLOQ), precision, recoveries and stability of the method were all validated. The specificity of the method was shown through comparing the chromatograms of blank plasma, blank plasma spiked with  $\alpha$ -obscurine and IS, and rat plasma sample after oral administration of the extracts of *L. japonicum* Thunb.

Calibration samples were prepared as follows: to 200  $\mu\text{l}$  of blank

rat plasma, 50  $\mu\text{l}$  of the internal standard solution and 20  $\mu\text{l}$  of the standard working solutions were added to yield final concentration ranges at: 0.354 to 14.16  $\mu\text{g/ml}$   $\alpha$ -obscurine in plasma. QC samples (0.4425, 2.832, 11.33  $\mu\text{g/ml}$ ) were independently prepared in the same manner for evaluation of accuracy, precision, and extraction recovery of the method.

The recoveries were calculated with the QC samples by comparing the peak areas of  $\alpha$ -obscurine in the spiked plasma samples with plasma-free samples containing the same amount of  $\alpha$ -obscurine.

Stability of  $\alpha$ -obscurine were tested in processed samples after storage at temperature for 24 h or frozen at -20°C for 2 weeks.

### Application to pharmacokinetic study in rats

Male Wistar rats (200 $\pm$ 20 g) were purchased from the Experimental Animals Center of China Medical University. They were kept in an environmentally controlled breeding room for 7 days before the start of the experiments, fed with a standard laboratory water and food. After the rats were given 5 g/kg extracts of *L. japonicum* Thunb. intragastrically, venous blood samples (0.5 ml each) were withdrawn to the heparinized tubes by eye puncture at 0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 12 and 24 h centrifuged at 4000 rpm (800 $\times$ g) for 10 min. The plasma samples were stored at -20°C until analysis.

### Data analysis

The plasma concentrations of the analytes at different times were expressed as mean $\pm$ standard deviation (SD) and the mean concentration-time curves were plotted. All data were processed by use of drug and statistics (DAS) software (version 2.0) for windows.

## RESULTS AND DISCUSSION

### LC method optimization

The wavelength was set at 250 nm. The optimal mobile phase consisted of a mixture of methanol:water:triethylamine (61:39:0.05, v/v/v).  $\alpha$ -obscurine, IS and endogenous interference in plasma sample solution were well separated. There are no interfering peaks caused by endogenous plasma components.

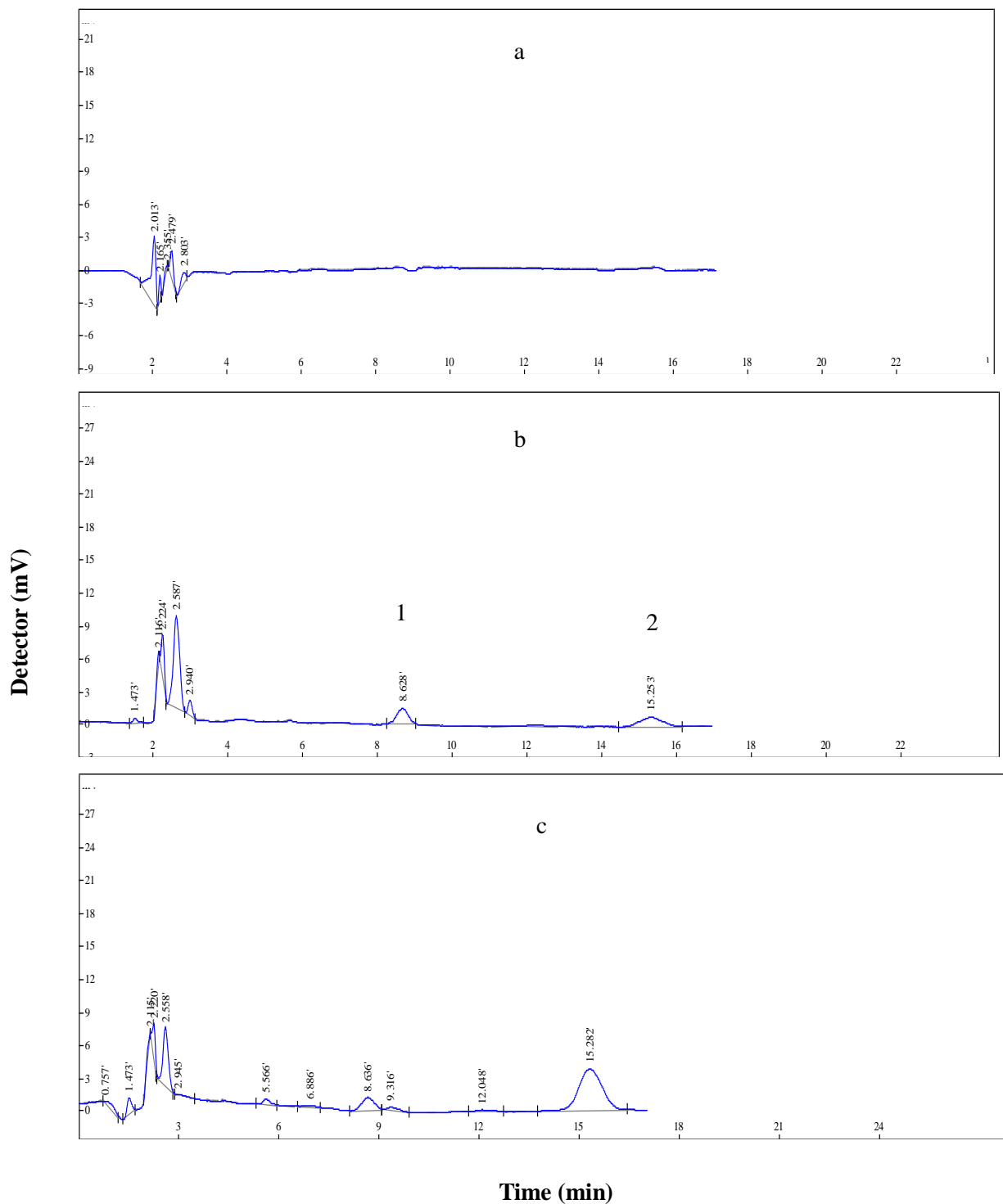
Due to the fact that  $\alpha$ -obscurine is such a molecule with double bonds, it should have ultraviolet absorption. Full UV spectra (200 to 400 nm) of sample were obtained by spectrophotometer. The UV absorption maximum of  $\alpha$ -obscurine was at 250 nm, which also gave a good absorption of the IS. Consequently, the wavelength was set at 250 nm.

Small amount of triethylamine were added into the mobile phase, because  $\alpha$ -obscurine is a natural weak alkaloid.

### Validation of the method

#### Specificity

Typical chromatograms of blank plasma, blank plasma spiked with  $\alpha$ -obscurine and IS, and rat plasma sample

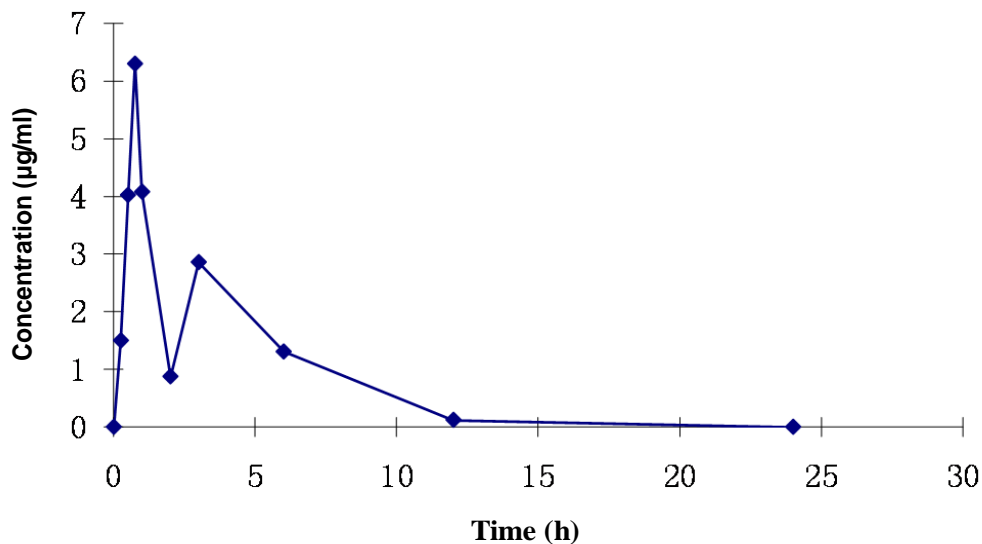


**Figure 2.** Typical chromatograms obtained from determination of  $\alpha$ -obscurine in plasma samples: (a) blank sample; (b) blank plasma spiked with  $\alpha$ -obscurine and IS; (c) a rat plasma sample 2.5 h after oral administration of Peaks: 1= IS and 2= $\alpha$ -obscurine.

after oral administration of the extracts of *L. japonicum* Thunb. are as shown in Figure 2. IS and  $\alpha$ -obscurine were eluted at 8.628 and 15.253 min, respectively.

#### Calibration plot and LLOQ

The calibration curve for the determination of  $\alpha$ -obscurine



**Figure 3.** Pharmacokinetic profiles of  $\alpha$ -obscurine after a single oral doses of 5 g/kg in rats.

**Table 1.** Precision, accuracy and stability of HPLC analysis of  $\alpha$ -obscurine in rat plasma samples (n=5).

Concentration ( $\mu\text{g/ml}$ )	Precision RSD (%)		Accuracy (%)	Stability RSD (%)	
	Intra-day	Inter-day	Intra-day	Room temperature for 24 h	Frozen for 14 days
0.4425	1.8	3.7	101.6 $\pm$ 4.3	1.4	2.4
2.832	2.6	2.4	96.03 $\pm$ 2.6	1.7	2.8
11.33	2.3	2.1	98.48 $\pm$ 3.8	3.8	2.0

in rat plasma was linear over the range 0.354 to 14.16  $\mu\text{g/ml}$ . Least squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The limit of quantification (LOQ) was 0.3  $\mu\text{g/ml}$ . The mean values of regression equation of the analytes in rats plasma were:

$$Y=2.299X+0.123(\gamma=0.9995).$$

### Precision and accuracy

The precision of the method was studied by estimation of intra- and inter-day relative standard deviations of values by determining LQC, MQC and HQC plasma samples. At least five QC samples were processed and injected on a single day (intra-day) and at different days (inter-day). The precision was evaluated by the relative standard deviation (RSD) of intra- and inter-day assay. The RSD of intra- and inter-day were  $\leq 2.6$  and  $\leq 3.7\%$ , respectively (Table 1).

Furthermore, as the sample process involved in extraction by ether, the average extraction and method recoveries of assay were 70.81 to 75.27% and 96.03 to 101.6%, respectively.

### Stability

Plasma samples left at room temperature for 24 h or frozen at  $-20^{\circ}\text{C}$  for 2 weeks were checked. The deviation of the mean test responses were within  $\pm 5\%$ , no effect on quantitative detection was observed. The results suggested that rat plasma samples containing  $\alpha$ -obscurine can be controlled under normal laboratory conditions. The results are shown in Table 1.

### Pharmacokinetic study of $\alpha$ -obscurine in rats

Due to the lack of an appropriate analytical method, there was no information about  $\alpha$ -obscurine before this study. By using this method for determining  $\alpha$ -obscurine in rats plasma, satisfactory results were obtained. It was successfully used for the pharmacokinetic study on extract of *L. japonicum* Thunb. The mean plasma concentration versus time profiles of  $\alpha$ -obscurine is as shown in Figure 3.

The peak of  $\alpha$ -obscurine in rat plasma occurred rapidly, which was approximately 0.75 h ( $T_{\text{max}}$ ) and it was eliminated from plasma with a MRT of 4.5 h. The values of  $T_{1/2}$  and  $C_{\text{max}}$  were 2.68 h and 6.306  $\mu\text{g/ml}$ , respectively.

The pharmacokinetic results are similar to Huperzine A with swift absorption ( $T_{1/2}$  1.4 h) (Yue et al., 2007). This was probably related to their similar structure, which both contain two N atoms and similar structure to pyridine ring.

## Conclusions

Generally, a simple and sensitive HPLC method had been established and validated to quantify  $\alpha$ -obscurine in rat plasma. To our knowledge, the method has not been reported earlier. The pharmacokinetic results are very useful for evaluating the clinical efficacy of *L. japonicum* Thunb.

$\alpha$ -obscurine medicine/curve appears in the form of double apices phenomena, respectively in oral administration methods after 0.25 and 3 h. According to the current research material, double apices phenomena may be by liver/intestinal material circulation, double part absorbed or stomach/bowel circulation, etc. Various causes may also be by several reasons of joint action of weak alkali medicine (morphine, diazepam) often in the gastrointestinal cycle.  $\alpha$ -Obscurine is an alkaline drug in which the petronas appear, and whether this is caused by gastrointestinal circulation remains to be further studied experimentally.

This paper only reports the pharmacokinetic studies after oral administration of ethanol extract of *L. japonicum* Thunb, the studies on the pharmacokinetic comparison of  $\alpha$ -obscurine and the ethanol extract are being tested in our laboratory.

## ACKNOWLEDGEMENT

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## Full Length Research Paper

# Antimicrobial and antioxidant activities of substituted halogenated coumarins

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Pathogens frequently display resistance to current drugs, which frequently lack selectivity/efficacy and have detrimental side effects. Thus, there is a constant need for novel therapeutic agents. Coumarins belong to the family of lactones, having a benzopyrone system that can be isolated from plants as well as total synthesis that can be carried out in the laboratory. To date, many chemical reactions have been established that can be used to synthesize coumarins. The synthesis of coumarins and their derivatives has attracted the attention of organic and medicinal chemists, as these are widely used as fragrances, pharmaceuticals and agrochemicals. In the present study, the antimicrobial and antioxidant activities of substituted coumarin analogue compounds have been screened. 3-(2-bromoacetyl)-2H-chromen-2-one (CMRN3) and 3-(2, 2-dibromoacetyl)-2H-chromen-2-one (CMRN6) showed bacterial growth inhibition for all the tested species except *Klebsiella pneumonia* and *Bacillus stearothermophilus*. CMRN4 and CMRN5 displayed moderate bacterial inhibition against *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*. CMRN3 and CMRN6 had a minimum inhibition concentration at 0.75 mg/ml against *B. cereus*, *Bacillus coagulans*, and *Streptococcus faecalis*. They displayed a minimum inhibitory concentration (MIC) of 1.5 mg/ml against *Escherichia coli* and *S. aureus*. (CMRN5) displayed an MIC at 0.75 mg/ml against *M. luteus* and 1.5 mg/ml against *S. aureus*. Compounds 3-(2-bromoacetyl)-6-chloro-2H-chromen-2-one (CMRN4), 3-(2-aminothiazol-4-yl)-6-bromo-2H-chromen-2-one (CMRN7), 3-acetyl-6-bromo-2H-chromen-2-one (CMRN1) exhibited potent antioxidant activity at 85, 61 and 56%, respectively, as evaluated by the DPPH free radical method.

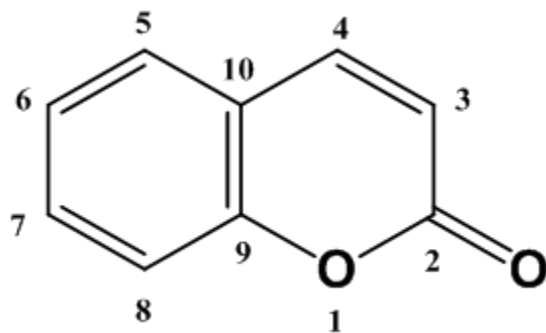
**Key words:** Coumarin, antibacterial, antifungal, antioxidant.

## INTRODUCTION

The synthesis of coumarins and their derivatives has attracted considerable attention from organic and medicinal chemists (Gacche and Jadhav, 2012; Paramjeet et al., 2012) because of the scope of possible beneficial effects on human health (Melagraki et al., 2009). The development of concomitant polymorphism in 3-acetyl coumarin also attracted chemists due to their potential application in various single crystal X-ray studies (Munshi et al., 2004).

Coumarins consist of a large class of phenolic substances

found in plants and are made of fused benzene and  $\alpha$ -pyrone rings (Venugopala et al., 2013), as represented in Figure 1 (Smyth et al., 2009). Coumarins were first synthesized in 1868 via the Perkin reaction, and many simple coumarins are still prepared through this method. In early 1900, the Knoevenagel reaction emerged as an important synthetic method to synthesize coumarin derivatives with carboxylic acid at the 3-position. Nowadays, different methods for synthesis of coumarins have been reported, including the Pechmann, Reformatsky and Wittig



**Figure 1.** Chemical structure of 2H-chromen-2-one.

reactions (Nikhili et al., 2012).

As substitutions can arise at any of the six available sites of their basic molecular moiety (1, 2-benzopyrone), these compounds are exceptionally variable in structure. This structural variety leads to compounds displaying multiple pharmacological and biological properties such as antibacterial (Creaven et al., 2006), antifungal (Basanagouda et al., 2010), anti-inflammatory (Melagraki et al., 2009) and antioxidant ( Abdel-Wahab et al., 2011) activities.

## MATERIALS AND METHODS

All the chemicals were obtained from Aldrich and Merck chemical company and were used without further purification. Reactions were monitored by thin layer chromatography (TLC). TLC was performed on Merck 60 F-254 silica gel plates with ethyl acetate and *n*-hexane (7:3) as solvent system and visualization with ultra violet (UV)-light or iodine chamber. Melting points were determined on a Büchi melting point B-545 apparatus. Liquid chromatography–mass spectrometry (LC-MS) was performed on an Agilent Technologies 1200 series instrument. Yields refer to isolated products. Synthesis of title compounds CMRN1 – CMRN7 is illustrated in Figure 2

### 3-Acetyl-6-substituted-2H-chromen-2-one (CMRN1 and CMRN2)

A mixture of 5-substituted salicylaldehyde (0.1 mol) and ethylacetoacetate (0.11 mol) were taken in a conical flask, stirred and cooled. To this mixture, 1 g of piperidine was added with shaking. The mixture was then maintained at freezing temperature for 2 to 3 h, and then a yellow coloured solid mass separated out. The lumps were broken in cold ethanol and filtered. The solid was washed with cold ethanol and dried which gave satisfactory yields of CMRN1 and CMRN2. The products were recrystallized from hot glacial acetic acid and formation of the products was confirmed by the difference in melting point and  $R_f$  values on thin layer chromatography (Chopra et al., 2006).

### 3-(2-Bromoacetyl)-2H-chromen-2-one (CMRN3), (CMRN4), (CMRN5)

To a solution of 3-acetyl-6-substituted-2H-chromen-2-one (0.1 mol) in 20 ml of alcohol free chloroform, bromine (0.1 mol) was added to

5 ml of chloroform, with intermittent shaking. The mixture was heated for 15 min on a water bath to expel most of the hydrogen bromide, cooled and filtered. The solid on washing with ether gave satisfactory result of almost pure product. The formation of compounds was confirmed by the difference in melting point and  $R_f$  values on thin layer chromatography (Chopra et al., 2007).

### 3-(2,2-Dibromoacetyl)-2H-chromen-2-one (CMRN6)

To a solution of 3-(2-monobromoacetyl)-2H-chromen-2-one (0.1 mol) in 20 ml of alcohol free chloroform, bromine (0.1 mol) was added to 5 ml of chloroform, with intermittent shaking. The mixture was heated for 15 min on a water bath to expel most of the hydrogen bromide, cooled and filtered. The solid on washing with ether gave satisfactory results of almost pure product. The formation of compound was confirmed by the difference in melting point and  $R_f$  values on thin layer chromatography (Chopra et al., 2007).

### 3-(2-Aminothiazol-4-yl)-6-bromo-2H-chromen-2-one (CMRN7)

Suspension of 6-bromo-3-(2-bromoacetyl)-2H-chromen-2-one (0.1 mol) in 17.5 ml of hot ethanol was treated with thiourea (0.11 mol) a mild exothermic reaction took place, leading to a clear solution that soon deposited as crystals. The deposit was removed, washed with ethanol and then boiled with water containing sodium acetate which yielded (80%) of 3-(2-aminothiazol-4-yl)-6-bromo-2H-chromen-2-one and the product obtained was recrystallized from absolute ethanol. The formation of compound was confirmed by the difference in melting point and  $R_f$  values on thin layer chromatography (Chopra et al., 2009).

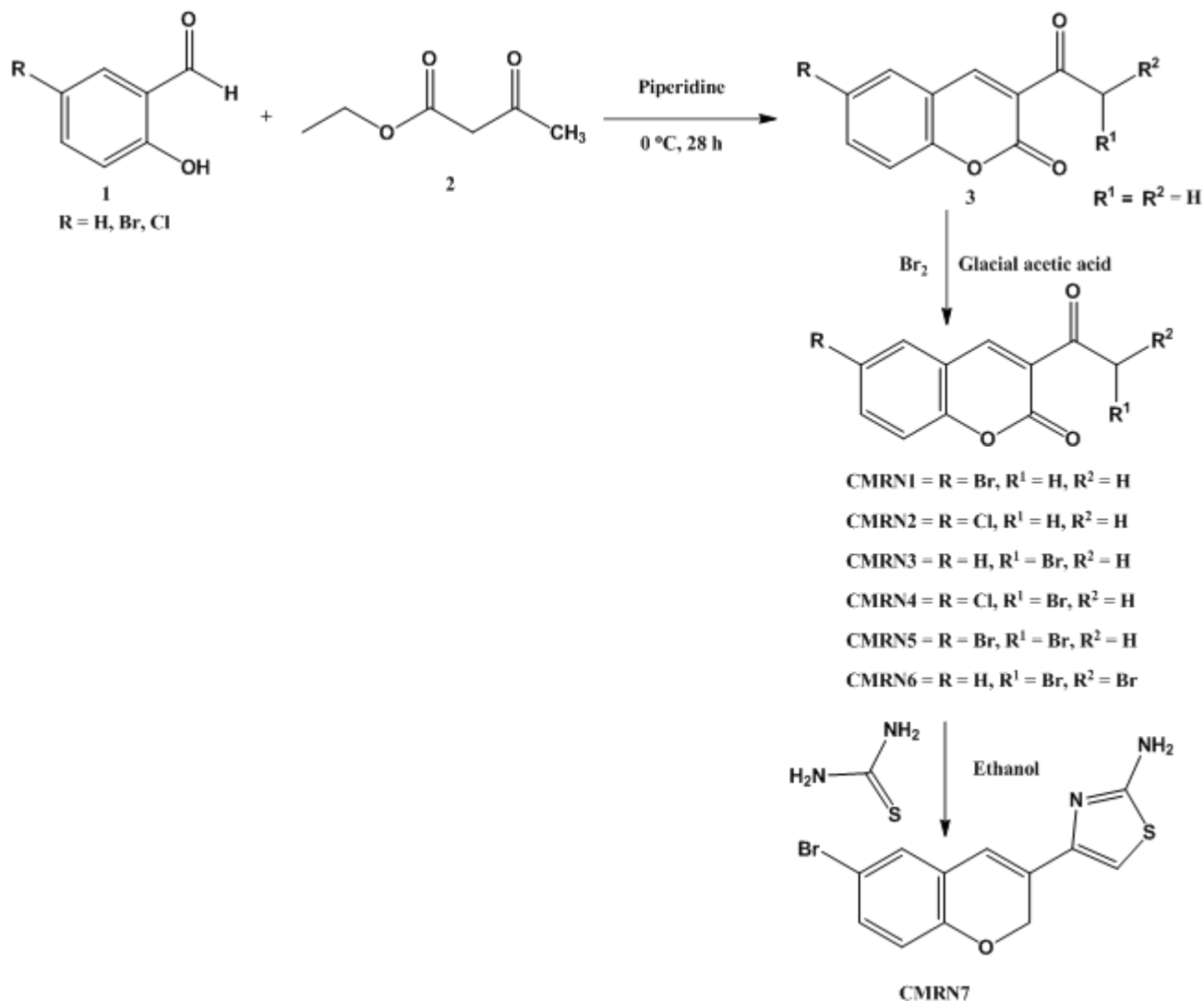
## Biological screening

The compounds were screened for their anti-bacterial, anti-fungal and anti-oxidant activity according to standard protocols.

### Antimicrobial activity

The development of antimicrobial resistance by many organism demands novel compounds from the pharmaceutical industry that are inexpensive and possess a broad spectrum activity. The antimicrobial activity and the minimum inhibitory concentration (MIC) of the compounds were carried out using the method of Cos et al. (2006) using the agar disc diffusion method. The bacterial strains used were based on the standard recommendations and were obtained from the stock collection in the Department of Biotechnology and Food Technology, Durban University of Technology. *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Streptococcus faecalis*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus stearothermophilus*, *Citrobacter freundii*, *Staphylococcus aureus*, and *Micrococcus luteus* were used in this study.

Cultures were plated out and verified. Stock cultures were stored in micro bank vials (Davies diagnostics, South Africa) using 50% glycerol. When needed they were plated out on Nutrient Agar (Biolab) plates and grown in nutrient broth (Biolab) for 24 h at 37°C. The concentrations of bacterial cells were adjusted to MacFarland standard of 0.5 absorbance which corresponded to  $10^8$  cfu/ml. A suspension (100  $\mu$ l of  $10^8$  cfu/ml) of the test bacteria was spread on Mueller Hinton Agar plates (Fluka, Biochemika). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper and they were dried in open sterile petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were inoculated with 10  $\mu$ l of sample at a concentration of 3 mg/ml and placed onto inoculated agar plates and incubated at 37°C for 24 h.



**Figure 2.** Schematic illustrating the pathway for the synthesis of substituted halogenated coumarins.

Each concentration was tested in triplicate. 100% dimethyl sulphoxide (DMSO) (10  $\mu$ l) was used as the negative control and ciprofloxacin 3 mg/ml (Fluka, Biochemika) was used as a positive control.

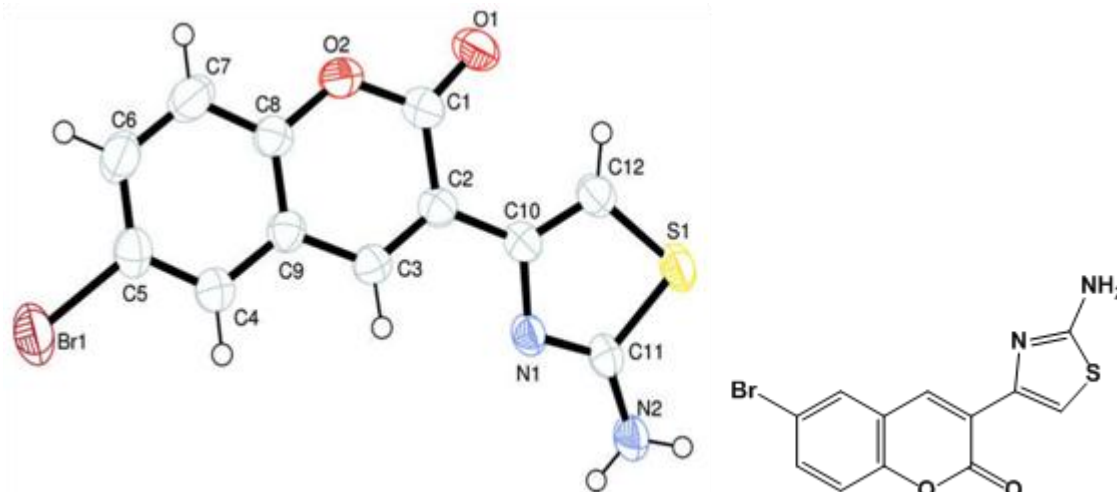
Preliminary disk diffusion assays were carried out in order to determine the level in inhibition of all compounds (CMRN1 to CMRN7) on the bacterial species. Where zones of inhibition equalled or exceeded 8 mm, these results were recorded and further analysis by MIC assay was conducted. The minimum inhibitory concentration (MIC) determination of the tested compounds was investigated with ciprofloxacin as a positive control. Dilutions of the test compounds (CMRN3, CMRN5 and CMRN6) were prepared in DMSO at the following concentrations 3 mg/ml, 1.5; 0.75; 0.37; 0.18 and 0.09 mg/ml. Ciprofloxacin was used as the positive control, tested at a concentration of 3 mg/ml against all bacterial species. All concentrations were tested against the bacterial species *B. cereus*, *B. coagulans*, *S. faecalis*, *E. coli* and *M. luteus*. MIC values for each compound against the bacteria tested are shown in Table 4.

The antifungal activity was evaluated on three yeast cultures, *Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae* and

two fungal species, *Aspergillus flavus* and *Aspergillus niger*. The yeast cultures were grown in Sabouraud dextrose broth for 24 h at 37°C. The fungi were incubated at 28°C for 4 to 7 days in Sabouraud Dextrose Agar until sporulation. The spores were collected in 10 ml sterile distilled water, counted in a Neubauer counting chamber and the concentration adjusted to 10<sup>6</sup> spores/ml. Sterile distilled water containing the fungal spores (10<sup>6</sup> spores/ml) was poured over the Sabouraud dextrose agar base plates (Biolab, Merck, South Africa). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper and they were dried in open sterile petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were inoculated with 10  $\mu$ l of sample at a concentration of 3 mg/ml and placed onto inoculated agar plates and incubated at 25°C for 24 h. Each concentration was tested in triplicate. 100% DMSO (10  $\mu$ l) was used as the negative control and whilst amphotericin B, 3 mg/ml (Fluka, Biochemika) was used as a positive control.

The effect of the compound was determined by measuring the diameter of clearing around the disks in mm. This zone of inhibition indicates the level of antimicrobial activity of the tested compound and the MIC value was the lowest concentration at which a zone of





**Figure 3.** The structure of the title compound drawn with 50% probability displacement ellipsoids.

inhibition was noticed.

#### Antioxidant activity

The radical scavenging activity of the compounds were measured using the stable free radical scavenger, DPPH (2, 2 diphenyl-2-picryl hydrate), decolouration assay described by (Choi et al., 2002). Stock solutions of the substituted coumarins analogues were made in methanol and were diluted to final concentrations of 1000, 500, 250, 100, 80, 60, 40, 20 and 1  $\mu\text{g/ml}$  in methanol. The results were compared with Quercetin-3-rutinoside which is a potent antioxidant. The radical scavenging was measured as the decolourization percentage of the test sample using the following formula:

$$\text{Scavenging capacity (\%)} = \frac{100 - (\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of negative control}} \times 100$$

## RESULTS AND DISCUSSION

### Characterization of CMRN7

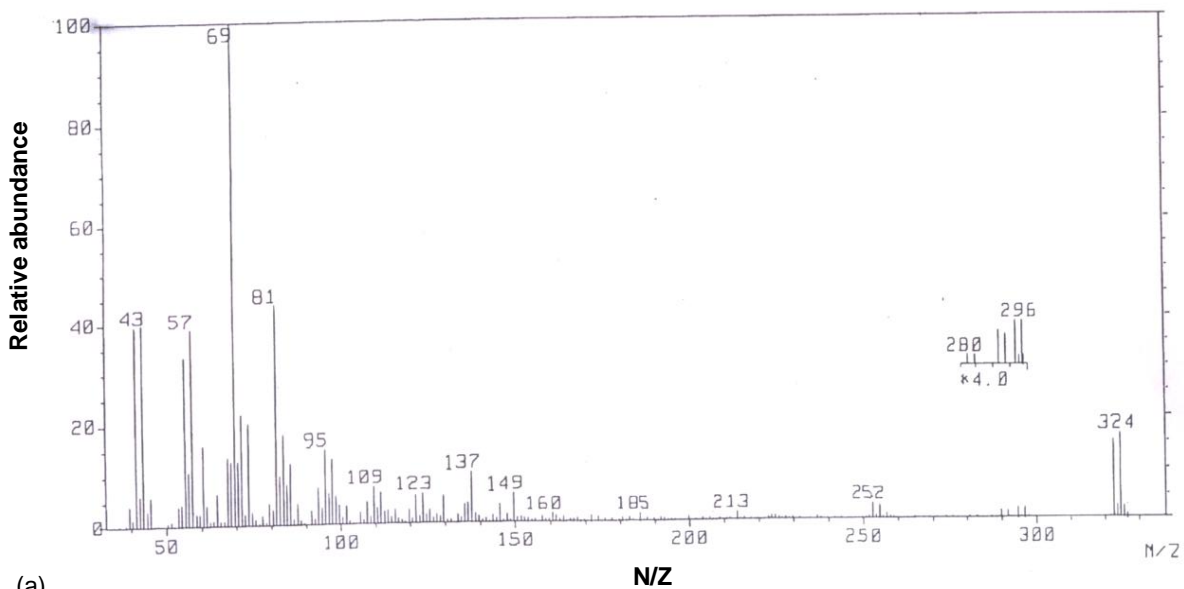
The crystal data of CMRN 7 and physicochemical characteristics of CMRN 1-7 are shown in Tables 1 and 2, respectively. The 50% probability displacement ellipsoids structure, mass spectrum and probable fragmentation pattern of CMRN 7 is shown in Figures 3, 4a and b, respectively. In the present study, the antimicrobial activities and the MIC values of a series of seven coumarins derivatives were screened for their antimicrobial activity using the broth micro dilution method against ten strains of bacteria. The results obtained depicted in Table 1 revealed that compounds 3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN3), 3-(2,2-dibromoacetyl)-2*H*-chromen-2-one (CMRN6), 6-bromo-3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN5) and 3-(2-bromoacetyl)-6-chloro-2*H*-chromen-2-one (CMRN4) exhi-

bited inhibitory effect on the growth of the tested strains *in vitro*. Thus, these compounds showed more or less pronounced antibacterial potencies, affecting both Gram-positive and Gram negative microorganisms.

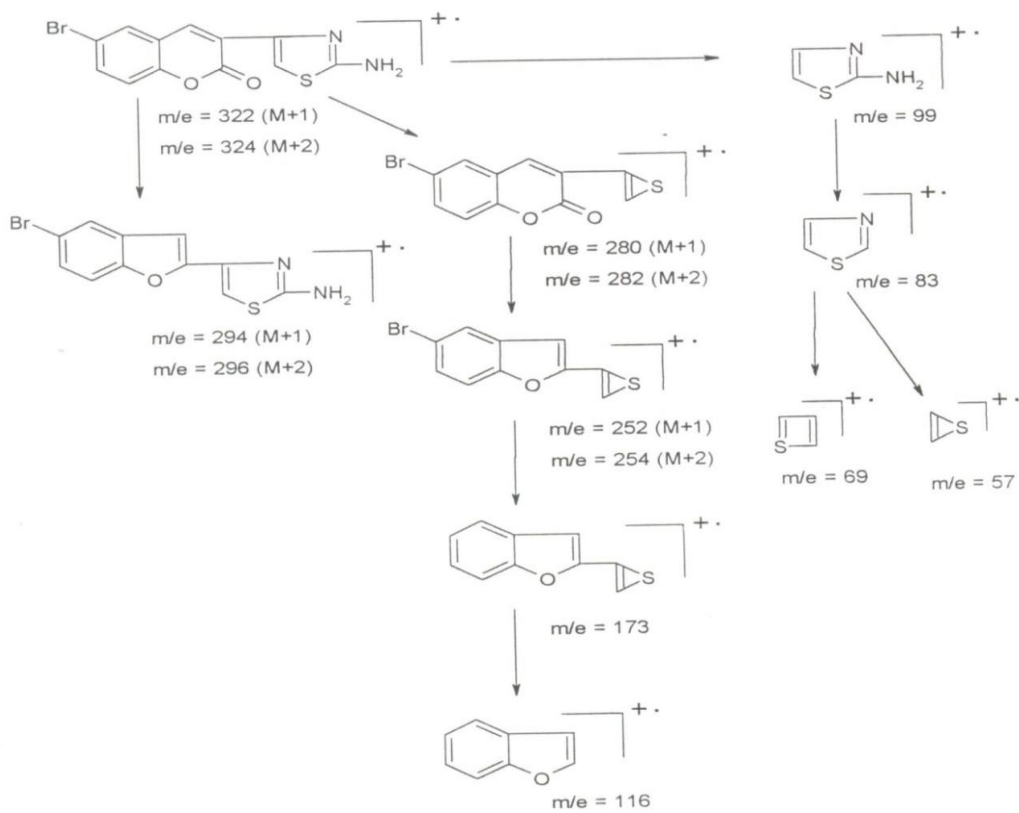
Among the active coumarin (Table 3), compounds 3-(2-Bromoacetyl)-2*H*-chromen-2-one (CMRN3) and 3-(2, 2-Dibromoacetyl)-2*H*-chromen-2-one (CMRN6) displayed bacterial inhibition growth to most of the tested microorganisms with the exception of *B. stearotherophilus* and *K. pneumonia*. The MIC was the lowest concentration of the tested compound that inhibited bacterial growth. It was observed that compound (CMRN3) and (CMRN6) had a minimum inhibition concentration (Table 4) of 0.75 mg/ml against *B. cereus*, *B. coagulans*, and *S. faecalis*. Compound CMRN5 showed an MIC of 0.75 mg/ml against *M. luteus*. Compounds (CMRN3) and (CMRN6) displayed an MIC of 1.5 mg/ml against *E. coli*, *S. aureus*, and CMRN5 displayed an MIC of 1.5 mg/ml against *S. aureus*.

This antibacterial activity was due to the incorporation of 3-(2-bromoacetyl) and 3-(2, 2-dibromoacetyl) on carbon three of the coumarin nucleus. Unpredictably the presence of bromide and chloride on carbon six of coumarin nucleus (Table 6) in compound CMRN4 and CMRN5 made the compounds exhibit less antibacterial activity against bacterial species tested. The substitution of the bromine on compound (CMRN6) and (CMRN3) at position 3 to the carbon atom at position 6 on the coumarin nucleus and the addition of 3-(2-aminothiazol-4-yl) on carbon 3 of the compound (CMRN7) contributed to a notable loss of antimicrobial efficiency. According to previous studies conducted by (Zavrsnik et al., 2011), a substitution in the para position of the compound's ring structure improves antibacterial activity. Our study indicates that when bromine is present in the ortho position of the coumarin compound's ring structure (Table

MASS SPECTRUM  
 Sample: COMPOUND D  
 RT: 0.18" EI (Pos.) GC 264.0c BP: m/z 69.0000 Int. 50.7142 LV 0.00  
 Scan# (10)



(a)



(b)

**Figure 4.** (a) LC-MS and (b) probable fragmentation pattern of 3-(2-aminothiazol-4-yl)-6-bromo-2H-chromen-2-one (CMRN 7).

**Table 1.** Crystal data and measurement details for CMRN 7.

Crystal data	CMRN 7
Radiation	Mo K $\alpha$
Crystal system	Monoclinic
Space group	<i>P</i> 2 <sub>1</sub> / <i>n</i>
<i>a</i> (Å)	7.031 (4)
<i>b</i> (Å)	13.804 (8)
<i>c</i> (Å)	12.453 (7)
$\alpha$ (°)	90
$\beta$ (°)	90.047 (9)
$\gamma$ (°)	90
Volume (Å <sup>3</sup> )	1208.6(12)

**Table 2.** Physicochemical characteristics of 3-mono/dibromoacetyl-6-halogenated coumarin analogues CMRN 1-7.

Code	MF (M. Wt.)	Yield (%)	mp. (°C)
CMRN 1	C <sub>11</sub> H <sub>7</sub> BrO <sub>3</sub> (265)	96	120-122
CMRN 2	C <sub>11</sub> H <sub>5</sub> Br <sub>3</sub> O <sub>3</sub> (421)	95	146-148
CMRN 3	C <sub>11</sub> H <sub>7</sub> BrO <sub>3</sub> (265)	98	220-222
CMRN 4	C <sub>11</sub> H <sub>6</sub> Br <sub>2</sub> O <sub>3</sub> (343)	95	204-206
CMRN 5	C <sub>11</sub> H <sub>6</sub> BrClO <sub>3</sub> (299)	94	180-182
CMRN 6	C <sub>11</sub> H <sub>7</sub> ClO <sub>3</sub> (222)	95	218-220
CMRN 7	C <sub>12</sub> H <sub>7</sub> BrN <sub>2</sub> O <sub>2</sub> S(321)	87	210-212

**Table 3.** Antimicrobial activity of substituted halogenated coumarin derivatives.

Bacteria	Zone of inhibition (mm)							Control ciprofloxacin
	CMRN1	CMRN2	CMRN3	CMRN4	CMRN5	CMRN6	CMRN7	
<i>B.cereus</i>	NA	NA	10	6	7	10	NA	29
<i>E.coli</i>	NA	NA	8	NA	NA	8	NA	35
<i>M.luteus</i>	NA	NA	6	7	8	6	NA	29
<i>S.aureus</i>	NA	NA	10	7	10	11	NA	25
<i>S.marcescens</i>	NA	NA	6	NA	NA	6	NA	35
<i>C.freundii</i>	NA	NA	6	7	NA	6	NA	30
<i>B.coagulans</i>	NA	NA	10	NA	7	10	NA	28
<i>B.stearothermophilus</i>	NA	NA	NA	NA	NA	NA	NA	30
<i>S.faecalis</i>	NA	NA	10	NA	NA	11	NA	22
<i>K.pneumoniae</i>	NA	NA	NA	NA	NA	NA	NA	36
<b>Yeast</b>								
<i>C.albicans</i>	NA	NA	NA	6	6	NA	NA	30
<i>C.utilis</i>	NA	NA	NA	6	6	NA	NA	28
<i>S.cerevisiae</i>	NA	NA	NA	NA	6	6	NA	28
<b>Fungi</b>								
<i>A.flavus</i>	NA	NA	NA	NA	NA	NA	NA	19
<i>A.niger</i>	NA	NA	NA	NA	NA	NA	NA	19

Data are mean  $\pm$  SD (n = 3); NA: no activity

**Table 4.** Minimum inhibition concentration of substituted halogenated coumarin derivatives (MIC).

Bacteria	MIC mg/ml		
	CMRN3	CMRN5	CMRN6
<i>B. cereus</i>	0.75	-	0.75
<i>E. coli</i>	1.5	-	1.5
<i>M. luteus</i>	-	0.75	-
<i>S. aureus</i>	0.75	1.5	1.5
<i>B. coagulans</i>	0.75	-	0.75
<i>S. faecalis</i>	0.75	-	0.75

**Table 5.** Antioxidant activity of the substituted halogenated coumarins.

Compound code	Concentration ( $\mu\text{g/ml}$ )								
	1	20	40	60	80	100	250	500	1000
CMRN1	7	8	10	13	16	18	30	45	56
CMRN2	6	7	8	9	15	16	17	18	23
CMRN3	4	6	7	8	9	10	12	14	28
CMRN4	6	17	23	29	30	34	56	72	86
CMRN5	12	14	16	17	18	20	22	23	25
CMRN6	24	26	28	30	31	32	33	34	36
CMRN7	14	21	25	26	27	30	40	46	61
- control	-	-	-	-	-	-	-	-	0
+ control (Rutin)	-	-	-	-	-	-	-	-	95

6), the antibacterial activity is still pronounced, but not to the same level obtained by (Zavrsnik et al., 2011), who obtained MIC's ranging from 0.03 to 0.001 mg/ml for all para substituted coumarin compounds tested against bacterial species.

*In vitro* antifungal effects of the investigated compounds were tested against three yeast cultures, *Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae* and two fungi, *A. flavus* and *A. niger*. It was observed that compound (CMRN5) was found to have slight activity against the three yeast species tested, compound (CMRN4) showed a slight activity against *C. albicans* and *C. utilis* and compound (CMRN6) displayed a slight activity against *S. cerevisiae*.

The results of the DPPH radical scavenging activities of the coumarin analogue are summarized in Table 5. The results obtained clearly indicate that some of the compounds showed considerable free radical-scavenging activities. The order of the reactivity was CMRN4 (86%) > CMRN7 (61%) > CMRN1 (56%). However, the remaining substituted coumarins analogue showed activity in the range of 23 to 36% at 1 mg/ml as compare to the Rutin which was 95%.

## Conclusion

The investigation of antimicrobial screening data reveals

that the synthesized compounds, particularly CRMN3 and CRMN6, showed antibacterial activity against both Gram-positive and Gram-negative bacteria. Based on results, functional substitutions on the benzene ring selectively enhance or decrease inhibition of coumarin activity and the bromine on the acetyl group at carbon three on the coumarin nucleus seems to be a highly significant factor in influencing the biological activity of the compound. Compounds CMRN4, CMRN7 and CMRN1 displayed considerable antioxidant activity, 86, 61 and 56%, respectively.

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