2.1. Pharmacological properties of hypericin

Agostinis et al. (1995) examined the photosensitized inhibition of protein kinases involved in growth factor signalling pathways by hypericin. These workers found that nanomolar concentrations of hypericin inhibit the protein tyrosine kinase activities (PTK) of the epidermal growth factor receptor and the insulin receptor, while being ineffective towards the cytosolic protein tyrosine kinases Lyn, Fgr, TPK-IIB and CSK.

Butterweck et al. (2001) examined the pharmacological and endocrine effects of *Hypericum perforatum* and hypericin after repeated treatment. These workers examined the antidepressant efficacy of extracts comparable to tricyclic antidepressants such as imipramine.

Wada et al. (2002) examined the effects of St. John's Wort and hypericin on cytotoxicity of anticancer drugs. The extract was examined for antiproliferative effects of anticancer drugs and the expression of MDR1 mRNA using HeLa and its MDR1-overexpressing subline.

Avato et al. (2004) analyzed antimicrobial properties of different extracts from St. John's Wort (SJW) like MeOH; petroleum ether; $CHCl_3$ and EtOAc from aerial parts of the plant against selected microorganisms. The growth inhibition was found only for Gram-positive bacteria, *B. subtilis* and *B. cereus* being the most susceptible to the tested drugs.

Van de Putte et al. (2005) examined the tumoritropic effect of hypericin in mouse and observed the major determinants of the accumulation and dispersion of hypericin in subcutaneously growing mouse tumours. It was found that rapid exponential decay in tumour accumulation of hypericin as a function of tumour weight occurred for each of the six tumour models evaluated, and a similar relationship was found between tumour blood flow and tumour weight.

Bublik et al. (2006) investigated hypericin and pulsed laser therapy of squamous cell cancer in vitro. They compared continuous wave and pulsed laser light at longer wavelengths for activation of the phototoxic drug hypericin in human cancer cells. Fluorescence confocal microscopy revealed membrane and perinuclear localization of hypericin in the SNU cells with membrane damage seen after excitation with visible light or two-photon irradiation.

Sauviat et al. (2007) examined that hypericin activates L-type Ca2+ channels in cardiac myocytes. The effects and the mode of action of hypericin (1) were studied, in the dark, on the action potential (AP) and the L-type Ca2+ channel of frog atrial heart muscle, using intracellular microelectrode and patch-clamp techniques, respectively. Hypericin decreased the cellular cGMP level by 69% in atrial myocytes.

Sosa et al. (2007) examined the topical anti-inflammatory effect of extracts and compounds from *Hypericum perforatum* L. The H. perforatum extracts provided a dose-dependent reduction of Croton-oil-induced ear oedema in mice, showing the following rank order of activity: lipophilic extract > ethylacetic fraction > hydroalcoholic extract (ID50 (dose that inhibited oedema by 50%) 220, 267 and >1000 microg cm (-2), respectively).

Hiquchi et al. (2008) investgated the actions of hypericin and St. John's Wort (SJW) in retinal neovascularization, using a mouse model of oxygen-induced retinopathy (OIR). Gavage administration of hypericin or SJW significantly inhibited the degree of retinal neovascularization, but did not affect the area of retinal vasoobliteration in a mouse model of OIR.

Roelants et al. (2009) performed the in vitro study of the photocytotoxicity of bathochromically-shifted hypericin derivatives. These authors investigated that whether a newly-synthesized series

of hypericin derivatives with a bathochromic shift shows promise for future photodynamic therapy (PDT) applications.

Chang and Wang (2010) studied that hypericin, the active component of St. John's wort, inhibits glutamate release in the rat cerebrocortical synaptosomes via a mitogen-activated protein kinase-dependent pathway. Experiments revealed that hypericin-mediated inhibition of glutamate release (i) results from a reduction of vesicular exocytosis, not from an inhibition of Ca2+-independent efflux via glutamate transporter; (ii) is not due to an alternation of nerve terminal excitability; (iii) is associated with a decrease in presynaptic N- and P/Q-type voltage-dependent Ca2+ channel activity; and (iv) appears to involve the suppression of mitogen-activated protein kinase pathway.

Bramanti et al. (2010) studied the effects of hypericin on the structure and aggregation properties of β-amyloid peptides. These authors determined the secondary structure of 1-40 β-amyloid peptides by Fourier-transform infrared spectroscopy (FTIR) and characterized the peptide photophysical properties before and after self-assembly by using intrinsic tyrosine steady-state and time-resolved fluorescence.

Solar et al. (2011) investigated that photoactivated hypericin induces downregulation of HER2 gene expression. These authors studied the effect of photoactivated hypericin on the expression of the human epidermal growth factor receptor 2 (HER2) oncogene at both the mRNA and the protein level in SKBR-3 and MCF-7 breast adenocarcinoma cell lines.

Mondon et al. (2011) studied MPEG-hexPLA micelles as novel carriers for hypericin, a fluorescent marker for use in cancer diagnostics. Studies of the biodistribution of Hy in Fisher rats revealed escape of nanosized micelles (<32 nm) from the mononuclear phagocyte system

and these Hy-loaded micelles showed maximal accumulation in tumors and demonstrated the best tumor/muscle contrast visible 3 h after injection in the rat model.

2.2. Analytical review of hypericin

Mulinacci et al. (1999) performed quantitative analysis of hypericin in *Hypericum Perforatum* L. extract using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) methods. The HPLC analysis of these samples were performed using C_{18} column using water of pH 3.2 using H_3PO_4 (component A), MeOH (component B) and CH_3CN (component C) using multistep linear solvent gradient: 0.1 min 88% A and 12% C; from 10 to 15 min 82% A and 18% C; 30 min 55% A and 45% C; from 35 to 42 min 55% B and 45% C. The TLC analysis was performed using TLC plates (pre-washed with mobile phase) using mobile phase (toluene: ethyl acetate: formic acid, 50: 40: 10) for sample elution after spotting.

Chi and Franklin (1999) determined hypericin in plasma using RP-HPLC with fluorescence end-point detection. Hypericin and dansylamide (internal standard) were extracted from plasma using solid-phase extraction (SPE). The standard curve was linear over the concentration range, 5-100 ng/ ml of plasma and average recovery was found to be 72.6%.

Mauri and Pietta (2000) separated and identified hypericin, hyperforin pseudohypericin and adhyperforin using HPLC coupled simultaneously to a diode array detector (DAD) and electrospray mass spectrometry (ESI-MS). The hypericin derivatives in different extracts of *H. perforatum* were also quantified using DAD and MS detectors.

Zotou and Loukou (2001) determined hypericin and pseudohypericin in extracts from *Hypericum Perforatum* L. and pharmaceutical preparations by liquid chromatography-fluorescence detection. The compounds were eluted using inertsil ODS-3 column by triethylammonium acetate: methanol: acetonitrile (5: 15: 80). The linearity of the method was 0.025-4 ng. μL^{-1} and limit of detection was 0.2 ng.

Rayes and Koda (2001) developed and validated a HPLC method for the simultaneous determination of hypericins and satablized hyperforin in St. John's Wort extract. The prepared samples were analyzed isocratically with C_{18} column using mobile phase acetonitrile and 0.3% v/v phosphoric acid (90:10, v/v). The standard curves were linear over the concentration ranges, 0.5-2.5 µg/ml (hypericin), 0.35-1.6 µg/ml (pseudohypericin) and 5-50 µg/ml (hyperforin).

Bauer et al. (2001) performed HPLC analysis of hypericin, hyperforin and pseudohypericin in human plasma with fluorescence and ultraviolet detection at 315/590 nm (ex/em). The chromatgraphic separation was achieved with a Lichrospher RP column (5 µm, 250×4.6 mm I.D.). The limit of quantification for hypericin, pseudohypericin was found to be 0.25 ng/ml and 10 ng/ml for hyperforin.

Pirker et al. (2002) determined simultaneously hypericin, hyperforin in human plasma and serum using liquid- liquid extraction (LLE), high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry. The liquid–liquid extraction (LLE) was peformed with *n*-hexane–ethylacetate (70:30, w/w) as mobile phase. The standard curve was linear over the concentration range, 8.4–28.7 ng/ml (hypericin) and 21.6–242.6 ng/ml (hyperforin). Recoveries were found to be 32.2 and 35.6% for hypericin and 100.1 and 89.9% for hyperforin.

Kazlauskas and Bagdonaite (2004) quantified active substances in St. John's Wort (*Hypericin perforatum* L.) by HPLC method. The flavonoids (rutin, quercetin, and isoquercetin) were identified using UV detection at 254 nm and hypericin at 590 nm. It was observed that larger quantities of rutin were found in leaves, while those of quercetin, isoquercetin and hypericin - in flowers of St. John's wort.

Riedel et al. (2004) simultaneously determined hypericin and hyperforin - the two main active ingredients of St. John's Wort (SJW) extract in human plasma. The limit of quantification was 0.05 ng/mL for hypericin and 0.035 ng/mL for hyperforin. The accuracy of the method varied between 101.9 and 114.2% and the precision ranged from 4.7 to 15.4% (S.D., batch-to-batch) for both analytes. The method was linear at least between 0.05 and 10 ng/mL for hypericin and between 0.035 and 100 ng/mL for hyperforin.

Chandrasekera et al. (2005) performed quantitative analysis of the major constituents of St John's wort with high-performance liquidchromatography-electrospray mass spectrometry (HPLC-ESI-MS). The separation of the flavonoids and glycosides was achieved within 35 min and that of the hypericins and hyperforin within 9 min. The linear response range in ESI-MS was established for each compound and all had linear regression coefficient values greater than 0.97.

Ruckert et al. (2007) simultaneously determined total hypericin (protopseudohypericin, pseudohypericin, protohypericin and hypericin) and hyperforin in *Hypericum perforatum* (St. John's wort) extracts and its preparations by HPLC with electrochemical detection. The determination of total hypericin and hyperforin in one step was achieved by exposing the samples to artificial daylight in amber glass vials.

Tatsis et al. (2008) studied 1H NMR determination of hypericin and pseudohypericin in complex natural mixtures by the use of strongly deshielded OH groups. These authors demonstrated that one-dimensional (1)H NMR spectra of hypericin and pseudohypericin, in Hypericum perforatum extracts show important differences in the chemical shifts of the hydroxyl groups with excellent resolution in the region of 14-15 ppm.

Ayan and Cirak (2008) determined contents of hypericin, pseudohypericin in some *Hypericin perforatum* L. species growing in Turkey. The lowest levels of hypericin and pseudohypericin were detected in leaves of *H. hyssopifolium*. [0.030 and 0.051 mg/g dry weight (DW), respectively] whereas flowers of *H. montbretii*. produced the highest levels of both hypericin forms (2.52 mg/g DW hypericin and 3.58 mg/g DW pseudohypericin).

Ciogli et al. (2010) analyzed enantiomerization barriers of hypericin and pseudohypericin using dynamic high-performance liquid chromatography on immobilized polysaccharide-type chiral stationary phases and off-column racemization experiments. Complementary variable temperature off-column (i.e., in solution) racemization experiments delivered DeltaG (enant)(#) = 97.1-98.0 kJ/mol (27-45 degrees C) for hypericin and DeltaG(enant)(#) = 98.9-101.4 kJ/mol (25-55 degrees C) for pseudohypericin.

Anyzewska et al. (2010) determined using HPLC method with spectrophotometric detection, levels of hypericins expressed as hypericin in the herbal substance of St. John's Wort, in capsules and tablets containing the extract of St. John's Wort, tablets containing powdered herb and in tincture and juice from fresh St. John's wort. The results obtained after evaluation showed that the daily dose of hypericins taken by a patient as infusions was 0.328 mg on average for herbs in sachets and in bulk form.

Cao et al. (2011) performed isolation and purification of series bioactive components from *Hypericum perforatum* L. by counter-current chromatography. The ethyl acetate extract was separated by using the solvent system composed of hexane-ethyl acetate-methanol-water (1:1:1:1 and 1:3:1:3, v/v) in gradient through both reverse phase and normal phase elution mode, yielding a naphthodianthrone compound, hypericin with HPLC purity about 95%.

2.3. Pharmacological properties of khellin

Abdel-Fattah et al. (1983) performed a double-blind study to understand the effect of khellin in psoriasis. These workers orally administered khellin to 10 patients and subsequently exposed to sunlight for 4 months. It was found that 8 cases out of 10 responded positively with variable degrees of clearance and there was no response of 10 controls.

Vedaldi et al., (1988) investigated the photochemotherapeutic effect of khellin used for the treatment of vitiligo and psoriasis. These workers studied the interactions both in ground and excited states between the drug and DNA in vitro to elucidate the mechanism of action. It was found that khellin formed a molecular complex with DNA in the dark and the drug photoconjugated covalently with the macromolecule using subsequent irradiation (365 nm) with a low rate of photobinding.

Ubeda and Villar (1989) examined the relaxant action of khellin on vascular smooth muscle. These workers investigated relaxant action on base line and on K+ and noradrenaline-induced contractile tensions in rat aorta smooth muscle and on spontaneous contractile activity of rat portal vein. It was observed that khellin relaxed these preparations with equal potency suggesting a non-specific inhibition of calcium flux, without any difference related to the specific calcium channels.

Ubeda et al. (1991) studied the effects of khellin on contractile responses and 45Ca2+ flux in rat isolated aortae. Khellin (10(-5)-3.2 x 10(-4) M) produced a concentration-dependent inhibition of noradrenaline (10(-6) M) and high K+ (80 mM)-induced contractions. At 3.2 x 10(-4) M, khellin increased cAMP levels and reduced 45Ca2+ influx in resting tissues and in tissues stimulated by noradrenaline (10(-5) M) and high K+ without affecting basal 45Ca2+ efflux or noradrenaline induced 45Ca2+ efflux.

El Naser et al. (1992) examined the hypocholesterolemic effect of khellin and methoxsalen in male albino rats. These workers studied clinical chemistry parameters to obtain information for possible drug toxicity and the drugs were evaluated in four weeks double-dose study and found that both drugs significantly lowered low density lipoprotein cholesterol, high density lipoprotein cholesterol and total cholesterol at 0.45 mg/100 mg b.wt. for khellin and 0.27 mg/100 g b.wt. for methoxsalen, per day with very low density lipoprotein cholesterol and triglycerides unchanged.

Di Stefano et al. (1996) investigated that pertussis toxin reverses the inhibition of the adenylyl cyclase system by khellin in HeLa cells. These authors found that pretreatment of HeLa cells with pertussis toxin reverses both the inhibition of NaF-stimulated adenylyl cyclase activity and the stimulation of GTPase by khellin alone and plus UVA light, as previously reported.

Borges et al., (1998) studied the photophysical properties and photobiological activity of the furanochromones visnagin and khellin. The results have shown that the magnitude of all the three rate constants out of S1 (radiative, kf; internal conversion, kic and intersystem crossing, kisc) for visnagin (VI) and khellin (KH) strongly depend on the solvent, namely on its hydrogen bonding ability and polarity.

Hofer et al., (2001) examined the results obtained in the treatment of vitiligo with oral khellin plus UVA (KUVA). The drug KUVA was administered at sometime during a14 year period to 28 patients [age range, 15-51 years] most with extensive generalized vitiligo of more than 6 months duration. Of 17 patients who had continued therapy for longer than 3 months, 7 (41%) had a good response after a mean of 194 treatments (range, 69-386 treatments) and a mean cumulative UVA dose of 2,036 J/cm2 (range, 690-4,411 J/cm2), whereas lower response grades were observed in the patients with lower treatment numbers.

De Leeuw et al., (2003) studied the treatment of vitiligo with khellin encapsulated in L-phenylalanin stabilized phosphatidylcholine liposomes in combination with ultraviolet light therapy. After a mean treatment period of 12 months (range 10-14 months) 72% of the treated locations had a repigmentation response of 50% to 100%. Repigmentation of 75-100% was achieved on the face in 63%, the back in 59%, the arms in 58%, the trunk in 57%, the legs in 56% and on the hands in 4% of the patients.

Umar and Eriksson (2009) performed the computational study of khellin excited states and photobinding to DNA. These authors reported a theoretical investigation of the formation and spectroscopic properties of the furan and pyrone monoadducts between the photosensitizer khellin and DNA base thymine.

Vanachayangkul et al., (2010) demonstrated that the extract of Ammi visnaga fruit and khellin prevent cell damage caused by oxalate in renal epithelial LLC-PK1 and Madin-Darby-canine kidney (MDCK) cells. Khellin extract (KE) (e.g. 100 microg/ml) significantly decreased lactate dehydrogenase (LDH) release from LLC-PK1 (Ox: 8.46+0.76%; Ox + 100 microg/ml KE: 5.41+0.94%, $p<0.001$) as well as MDCK cells (Ox: 30.9+6.58%; Ox+100 microg/ml KE: 17.5+2.50%, $p<0.001$), which indicated a prevention of cell damage.

De Leeuw et al. (2011) investigated the treatment of vitiligo with khellin liposomes, ultraviolet light and blister roof transplantation. These authors evaluated the additional value of combining blister roof transplantation (BRT) with khellin in liposomes and ultraviolet light (KLUV) in the treatment of recalcitrant vitiligo patches.

Abu-Hashem and Youssef (2011) synthesized some new visnagen and khellin furochromone pyrimidine derivatives and investigated their anti-inflammatory and analgesic activities. These

workers synthesized 6-[(4-Methoxy/4,9-dimethoxy)-7-methylfurochromen-5-ylideneamino]-2-thioxo-2,3-dihydropyrimidin-4-ones 1a,b by the reaction of 6-amino-2-thiouracil with visnagen or khellin, respectively.

2.4. Analytical review of khellin

El-Yazigi and Said **(**1980) determined khellin in human serum and urine by high-performance liquid chromatography (HPLC) using theophylline as the internal standard. The chromatographic separation was performed on a C18 with 65% (v/v) methanol as the mobile phase. These workers analyzed khellin concentrations down to 100 ng/ml with run time of <20 min.

Abdel-salaam et al., (1985) spectropluorimetrically determined khellin and visnagin in fruits and liquid extract of *Ammi visnaga*. Khellin was estimated fluorimetrically in chloroform, whereas visnagin exhibited no contribution and estimated by measuring its fluorescence in ethanol, where khellin showed no interference. The method was found to be highly selective and the sensitivity range was 1-5 µg/mL for each compound.

Franchi et al., (1985) performed HPLC analysis to determine the contents of furanochromones, khellin and visnagin in various organs of *Ammi visnaga* (L.) Lam. at different developmental stages. These workers found that unripe fruits are the richest in both chromones, but the collection of ripe dry fruits seems more reasonable because they might not undergo degradation processes during desiccation and storage.

Abounassif et al., (1990) individually determined khellin, phenobarbitone and dipyrone in tablets by HPLC with a reverse phase column using methanol: water (68:32) as mobile phase at a flow rate of 0.7 ml/min and at a detection of 254 nm. The standard curve was linear over the concentration range, 0.5-4 µg/ml of khellin, 2.5-12.5 µg/ml of dipyrone and 1-7 µg/ml of phenobarbitone at a sensitivity of 0.01 AUFS with standard deviations of less than 2%. For khellin, dipyrone and phenobarbitone added to tablets, the mean recoveries +/- SD were found to be 101.0 +/- 0.65, 100.0 +/- 0.74 and 99.9 +/- 0.74, respectively.

El-Domiaty (1992) performed an improved HPLC analysis to determine khellin and visnagin in Ammi visnaga fruits and pharmaceutical formulations using an internal standardized technique. The chromatgraphic separation was achieved with a reverse-phase C18 column using a mobile phase water: methanol: acetonitrile (49:49:2) at a flow rate of 1.5 mL/min and analyzed with a spectrophotometer set at 250 nm. In this method, khellin was quantified in various pharmaceutical dosage forms, such as ampules, tablets, and suppositories, with relative standard deviations of 1.2, 1.4, and 1.7%, respectively

Carlin et al., (1993) performed gas chromatography (GC) analysis to separate and determine khellin in human serum. After extraction from chloroform, khellin and trioxalen, an internal standard, were separated from endogenous substances using a DB-17 capillary column. Spiked serum samples in the range 0.11–1.1 μg/ml were assayed and a linear calibration curve was obtained.

Zgorka et al., (1998) determined furanochromones and pyranocoumarins in drugs and *Ammi visnaga* fruits by combined solid-phase extraction–high-performance liquid chromatography (SPE-HPLC) and thin-layer chromatography–high-performance liquid chromatography (TLC-HPLC).

Günaydin and Erim (2002) determined khellin and visnagin in Ammi visnaga fruits by capillary electrophoresis. The micellar electrochromatographic separation of khellin and visnagin was performed using 10 mmol/l borate, 50 mmol/l sodium dodecylsulfate, 25% (v/v) acetonitrile as running buffer with a pH of 9.. The limits of detection for khellin and visnagin were found to be 2.36 and 1.97 μg/mL respectively using coumarin as internal standard for quantitation with UV detection at 245 nm.

Mawatari et al., (2003) fluorometrically determined khellin in human urine and serum by high-performance liquid chromatography using postcolumn photoirradiation. These workers performed separation of khellin and visnagin on a capcell pak C8 column using mobile phase 40% (v/v) ethanol containing 75 mmol l-1 H_2O_2 and postcolumn reagent,70 mmol l-1KH_2PO_4-NaOH buffer (pH 12.7) containing 50% (v/v) ethanol, were mixed with the mobile phase. The calibration curve of khellin was linear over the range of 65-2620 ng ml-1.

Fersi and Platz (2005) performed nanosecond time-resolved infrared studies of visnagin and khellin triplets and radical ions. No related neutral radicals by TRIR spectroscopy upon laser flash photolysis (LFP) of khellin in the presence of hydroquinone were found, but evidence for the formation of semiquinone and neutral visnagin radicals upon LFP of visnagin and hydroquinone were found.

El-Gogary and Grabner (2006) investigated the ultraviolet photoionization of the photosensitizer's khellin and visnagin in aqueous solution and in micelles. The results did not support the hypothesis of a significant role of one-photon ionization, the upper limits of the quantum yields of radical cation formation being phi < 0.01 for visnagin and phi < 0.004 for khellin.

Vanachayangkul et al., (2009) determined visnagin in rat plasma by liquid chromatography with tandem mass spectrometry and its application to in vivo pharmacokinetic studies. The mobile phase consisted of water and methanol (15:85, v/v) containing 0.1% formic acid and 5mM ammonium acetate. The linear standard curve ranged from 1.0 to 5000 ng/mL and the precision and accuracy (inter- and intra-run) were within 4.5% and 4.3%, respectively.

2.5. Pharmacological properties of vincristine and vinblastine

Douglas and Richard (1973) studied the interaction of vinblastine, vincristine and colchicine with serum proteins. It was also found that CLC was 19% more bounded to plasma than serum and the extent of adsorption for all three alkaloids was approximately proportional to both the free alkaloid concentration and the serum protein concentration. The per cent of drug bound to serum protein was about 75 per cent for VLB and VCR and 50 per cent for CLC at physiologic concentrations of protein.

Mareel and De Brabander (1978) examined the effect of microtubule inhibitors on malignant invasion in vitro. These workers observed that the malignant C3H/3T3 mouse cells MO4 invaded embryonic chick heart fragments in an organotypic coculture system on semisolid medium, which mimicked malignant invasion. In this system, at a dose of 1 microgram/ml, the microtubule inhibitors colchicine, demecolcine, vincristine sulfate, vinblastine sulfate, or methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-1-yl]-carbamate (Nocodazole) totally inhibited malignant invasion.

Takuma et al., (1982) observed ultrastructural changes of secreting rat-incisor ameloblasts after administration of vincristine and vinblastine. These workers further observed that polarity of nuclei and cytoplasmic organellae was remarkably disturbed; consequently, the regular secreting orientation of cellular products was altered greatly, leading to heterotopic deposition of large amounts of enamel matrix.

Van Belle et al., (1991) investigated the cellular pharmacokinetics of vinblastine and other vinca alkaloids in MO4 cells. It was observed that the ratio of accumulation, expressed as the ratio of intracellular over extracellular vinblastine, was dose-dependent and an initial accumulation level

was reached within 30 min and a second dose-induced level after 48 hours which remained constant for another 5 days. For vincristine and vindesine, intracellular accumulation was also seen but the process was less dose-dependent and the maximum level was reached after 6 hours.

Boman et al., (1995) examined that the encapsulation of vincristine in loposomes reduces its toxicity and improves its anti-tumor efficacy. These workers observed that vincristine can be encapsulated into large unilamellar vesicles in response to a transmembrane pH gradient with trapping efficiencies approaching 100%.

Tiburi et al., (2002) examined the comparative genotoxic effect of vincristine, vinblastine and vinorelbine in somatic cells of Drosophila melanogaster. These workers investigated genotoxicity of vinca alkaloids vincristine (VCR), vinblastine (VBL) and vinorelbine (VNR) in the wing Somatic Mutation and Recombination Test (SMART) of Drosophila. It was also found that VNR was able to induce, respectively, approximately 13.0 and 1.7 times more mutant clones per millimolar exposure unit as their analogues VCR and VBL.

Modriansky and Dvorak (2005) studied the microtubule disruptors and their interaction with biotransformation enzymes. It was found that microtubules disarray restricts the signaling by these two nuclear receptors regardless of cell cycle phase. Consequently, intact microtubules play an important role in the regulation of expression of cytochromes P450, which are under direct or indirect control of the two nuclear receptors.

Upreti et al., (2008) worked on identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance. These workers observed that vinblastine and other microtubule inhibitors characteristically promote the

phosphorylation of the key anti-apoptotic protein, Bcl-xL but putative sites of phosphorylation were inferred based on potential recognition by JNK, and no direct biochemical analysis was performed.

Noble et al., (2009) investigated the characterization of highly stable liposomal and immunoliposomal formulations of vincristine and vinblastine. It was observed that nanoliposome formulations of vincristine and vinblastine demonstrated similar pharmacokinetic profiles for the liposomal carrier, but increased clearance for liposome encapsulated vinblastine (t (1/2) = 9.7 h) relative to vincristine (t (1/2) = 18.5 h).

Arias et al., (2010) investigated that catharanthine alkaloids are noncompetitive antagonists of muscle-type nicotinic acetylcholine receptors. It was found that catharanthine alkaloids: (a) inhibited, (+/-)-epibatidine-induced Ca(2+) influx in TE671-halpha1beta1gammadelta cells with similar potencies (IC(50)=17-25microM), (b) inhibited [(3)H]TCP binding to the desensitized Torpedo AChR with higher affinity compared to the resting AChR, and (c) enhanced [(3)H]cytisine binding to resting but activatable Torpedo AChRs.

Banoczi et al., (2010) examined the **s**ynthesis and in vitro antitumor effect of vinblastine derivative-oligoarginine conjugates. These workers observed the coupling of vinblastine through its carboxyl group at position 16 with the N-terminal amino function of L-Trp methyl ester and found that after hydrolysis of the ester group, 17-desacetylvinblastineTrp was conjugated to the N-terminal amino group of oligoarginine via the C-terminal carboxyl group of the Trp moiety in solution and the antitumor effect of conjugates was studied in vitro on sensitive and resistant human leukemia (HL-60) cells.

Chen et al., (2011) examined that the activation of AMP-activated protein kinase is involved in vincristine-induced cell apoptosis in B16 melanoma cell. These workers found that vincristine induces AMPK activation (AMPKα, Thr 172) and Acetyl-CoA carboxylase (ACC, Ser 79) (a downstream molecular target of AMPK) phosphorylation in cultured melanoma cells in vitro.

2.6. Analytical review of vincristine and vinblastine

Dine et al., (1991) investigated the stability and compatibility studies of vinblastine, vincristine, vindesine and vinorelbine with PVC infusion bags. These workers developed an isocratic technique for the analysis of vinca alkaloids (vinblastine, vincristine, vindesine and vinorelbine) in parenteral solutions using high-performance liquid chromatography (HPLC) with UV detection and an Intersphere CN column. It was found that no significant drug loss was observed during simulated infusions (n = 4) for 2 h using PVC infusion bags and administration sets. Nagy-Turak and Vegh (1994) demonstrated the extraction and *in situ* densitometric determination of alkaloids from *Catharanthus roseus* by means of overpressured layer chromatography on amino-bonded silica layers I. These workers developed a simple, rapid and efficient method for the separation and spectrodensitometric determination of bis-indole alkaloids, minor components of *Catharanthus roseus*. The peak purity test and validation data confirmed that the method was sufficient for separation of these closely related alkaloids.

Singh et al., (2000) performed simultaneous HPLC analysis of vincristine, vinblastine, catharanthine and vindoline using a Bondapak C18 reversed-phase column, 10 µm (30 cm X 3.9 mm I.D.). The mobile phase was composed of acetonitrile: 0.1 M phosphate buffer: glacial acetic acid (38: 62: 0.3) with pH 4.14. The linearity was in the range 0.25µg-25µg for vincristine, vinblastine, catharanthine and vindoline and the recoveries of these compounds were found to be 97, 97, 96, 97 % respectively.

Tikhomiroff and Jolicoeur (2002) performed high-performance liquid chromatography (HPLC) with photodiode array and fluorescence detection for the screening of *Catharanthus roseus* secondary metabolites. The separation was achieved on a reversed-phase C 18 column for both

methods. In the first method, the separation of catharanthine, serpentine, tabersonine, vindoline, vinblastine, and vincristine was achieved in 20 min. whereas in the second method, ajmalicine, tryptophan, tryptamine and secologanine were separated in 13 min.

Barthe et al., (2002) determined vinca alkaloids by nonaqueous capillary electrophoresis with diode array detection. These workers separated 11 alkaloids (catharanthine, vinorelbine, anhydrovinblastine, vinflunine, vindoline, 4-O-deacetylvinorelbine, 4-O-deacetylvinflunine, vindesine, vinblastine, 4′-deoxy-20′, 20′-difluorovinblastine, vincristine) within a runtime of 10 min. The compounds were separated using a capillary of 38 cm effective length, a running buffer composed of 50 mM ammonium acetate and 0.6 M acetic acid in a methanol–acetonitrile (75:25, v/v) mixture.

Paci et al., (2003) performed quantitative analysis of vinca-alkaloids in chemotherapeutic infusion bags prepared in a hospital pharmacy using high-performance thin-layer chromatographic (HPTLC). The four compounds were separated using the solvent system CH_2Cl_2–CH_3OH (93:7, v/v). Vincristine (VCR) and vinorelbine (NVB) were assessed in the same run whilst vinblastine (VLB) and vindesine (VDS) were analyzed in a second run.

Gupta et al., (2005) simultaneously determined vincristine, vinblastine, catharanthine, and vindoline in leaves of catharanthus roseus by high-performance liquid chromatography with a mobile phase of acetonitrile & ndash: 0.1M phosphate buffer containing 0.5% glacial acetic acid (21:79, v/v; pH 3.5). The method was found to be selective and linear for alkaloid concentration in the range 0.25 µg & ndash: 25 µg/mL. The percent recovery of the alkaloids was found to be 96%, 97%, 98%, and 98%, respectively.

Schmidt et al., (2006) determined vincristine in infant plasma by liquid chromatography-atmospheric pressure chemical ionization-mass spectroscopy (APCI-MS). After solid-phase

extraction (SPE) of the sample, the lower limit of quantitation (LLOQ) was 0.18 ng/ml, the lower limit of detection was 0.09 ng/ml, and the linear calibration range was 0.18–180 ng/ml.

Hisiger and Jolicieur (2007) determined catharanthus roseus alkaloids vincristine, vinblastine by HPLC. These workers reviewed diverse considerations that are crucial to the efficiency of secondary metabolites separation and identification steps, such as biomass manipulation, extraction phase and protocols, HPLC separation and analysis protocols.

Ramirez et al., (2007) analyzed vinblastine, desacetylvinblastine and vincristine in human plasma using liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS. The calibration curves were linear across the range of 0.51–4.00 ng/ml (0.63–4.93 n*M*) for vinblastine, 0.74–3.93 ng/ml (0.96–5.11 n*M*) for desacetylvinblastine and 0.30–3.95 ng/ml (0.36–4.79 n*M*) for vincristine.

Shams et al., (2009) performed isolation and characterization of antineoplastic alkaloids from Catharanthus roseus L. Don. cultivated in Egypt by several chromatographic techniques. Vinblastine and vincristine, were isolated by the use of vacuum liquid chromatographic column on silica gel: aluminium oxide (1:1) mixed bed vacuum liquid chromatography (VLC), Charcoal column, and finally purified by centrifugally accelerated radial chromatography (Chromatotrone).

Guilhaumou et al., (2010) developed and validated an electrospray ionization LC/MS/MS method for quantitative analysis of vincristine in human plasma samples in order to investigate pharmacokinetics in a paediatric population. The limit of quantification was 0.25 ng/ml with a precision of 10.2% and an accuracy of 99.6%. The calibration curve was linear up to 50.0 ng/ml. Intra-day precision and accuracy ranged from 6.3% to 10% and from 91.9% to 100.8%,

respectively. Inter-assay precision and accuracy ranged from 3.8% to 9.7% and from 93.5% to 100.5%, respectively.

Siddiqui et al., (2011) simultaneously determined secondary metabolites from *Vinca rosea* plant extractives by reverse phase high performance liquid chromatography. These workers performed quantitative analysis of *V. rosea* plant extracts on a C 18 column by RP-HPLC equipped using mobile phase methanol: acetonitrile: ammonium acetate buffer (25 mM) with 0.1% triethylamine (15:45:40 v/v). The relative standard deviation (R.S.D.) was found to be than 2.68% and the recoveries were in the range of 98.09%-108%.

Chen et al., (2011) simultaneously determined vinblastine and its monomeric precursor's vindoline and catharanthine in *Catharanthus roseus* by capillary electrophoresis–mass spectrometry (CE-MS). Quantification of three components was assigned in positive-ion mode at a protonated molecular ion $[M+H]^+$. The detection limits of VLB, catharanthine and vindoline were found to be 0.8, 0.1 and 0.1 μg/mL, respectively. The precision was found to be less than 4.54% and the mean recovery of the analytes was 95.04–97.04%.

2.7. Pharmacological properties of strychnine and brucine

Fujisaki et al., (1994) examined strychnine and brucine as the potent inducers of drug metabolizing enzymes in rat liver: different profiles from phenobarbital on the induction of cytochrome P450 and UDP-glucuronosyltransferase. It was found that the administration of strychnine in the drinking water to rats significantly increased the hepatic microsomal activities of benzphetamine N-demethylation, strychnine 2-hydroxylation and testosterone hydroxylations at positions 16 alpha and 16 beta.

Zhao et al., (1997) studied the effect of brucine i.p. at analgesic doses on the nonspecific immune responses in normal and cyclophosphamide (Cyc)-treated mice. It was found that in normal mice, Bru slightly enhanced the clearance of charcoal particles, the phagocytosis of PMO, IL-1 production, the immune organ weights and the WBC counts ($P > 0.05$), whereas in Cyc-induced subnormal immunity model mice, Bru greatly enhanced these nonspecific immune responses ($P < 0.05$ or $P < 0.01$).

Tripathi and Chaurasia (2000) investigated the interaction of *Strychnos nux-vomica*-products and iron: with reference to lipid peroxidation. These workers studied the effect of strychnine, a pure alkaloid isolated from the seeds of S.nux-vomica and alcoholic extracts of the plant on $FeSO_4$-induced lipid peroxidation, hydroxyl radicals and superoxide radicals. Further, EDTA and strychnine showed 38% chelation of Fe^{2+} in 2 minutes and, under similar conditions, desferrioxamine showed only 3% chelation of Fe^{2+} and 21% for Fe^{3+} ions.

Miyakawa et al., (2002) investigated the glycine receptor antagonist, strychnine which blocked NMDA receptor activation in the neonatal mouse neocortex. These workers examined the contribution of glycine receptor GlyRs to Ca(2+) influx via NMDARs in neonatal mouse cortical

neurons. The GlyR antagonist, strychnine, was more effective in suppressing postsynaptic Ca (2+) influx than the GABA (A) R antagonist, picrotoxin, suggesting greater potentiation of NMDARs by GlyRs than by GABA (A) Rs.

Zlotos et al., (2003) studied the bisquaternary dimers of strychnine and brucine, which is a new class of potent enhancers of antagonist binding to muscarinic M 2 receptors. These workers synthesized the bisquaternary dimers of strychnine and brucine and examined their allosteric effect on muscarinic acetylcholine M (2) receptors. It was observed that these compounds retarded the dissociation of the antagonist [(3) H] N-methylscopolamine ([(3)H]NMS) from porcine cardiac cholinoceptors resulted in ternary complex formation.

Yin et al., (2003) studied the analgesic and anti-inflammatory properties of brucine and brucine N-oxide extracted from seeds of Strychnos nux-vomica. These workers observed that both brucine and brucine N-oxide revealed significant protective effects against thermic and chemical stimuli in hot-plate test and writhing test. However, on different phases they exerted analgesic activities in formalin test.

Wang et al., (2004) performed the RP-HPLC analysis of toxic alkaloids strychnine and brucine from postmortem specimens, which is a case report of fatal intoxication. The limit of detection was found to be 0.5 ng/mL blood for strychnine and brucine, and the limit of quantitation was 5 ng/mL blood for strychnine and brucine.

Deng et al., (2006) studied the apoptotic effect of brucine from the seed of Strychnos nux-vomica on human hepatoma cells is mediated via Bcl-2 and Ca2+ involved mitochondrial pathway. Brucine, among the four alkaloids, exhibited the strongest toxic effect, the mechanism

of which was found to cause HepG2 cell apoptosis, since brucine caused HepG2 cell shrinkage, the formation of apoptotic bodies, DNA fragmentation, cell cycle arrest, as well as phosphatidylserine externalization.

Deng et al., (2006) examined the anti-tumor effects of four alkaloids: brucine, strychnine, brucine N-oxide and isostrychnine from the seeds of Strychnos nux-vomica on HepG2 cells and its possible mechanism using MTT assay. These workers observed that brucine, strychnine and isostrychnine significantly inhibitory effects against HepG2 cell proliferation, whereas brucine N-oxide didn't have such an effect.

Jensen et al., (2006) studied the pharmacological characterization of tertiary and quaternary analogues as well as bisquaternary dimers of strychnine and brucine at human alpha1 and alpha1beta glycine receptors and at a chimera consisting of the amino-terminal domain of the alpha7 nicotinic receptor (containing the orthosteric ligand binding site) and the ion channel domain of the 5-HT3A serotonin receptor. Compared to strychnine and brucine, majority of the analogues displayed significantly increased Ki values at the glycine receptors and only few retained the high antagonist potencies of the parent compounds.

Hou et al., (2011) investigated the pharmacokinetics of brucine in rats after intravenous administration of liposomes containing total alkaloids from seed of Strychnos nux-vomica. It was observed that the ratios of brucine to total alkaloids in liposomes hardly varied with phospholipids composition and after comparing with SPC liposome, AUC of brucine was increased 13.3-fold whereas apparent volume of distribution was decreased to only 3.6% following intravenous administration of HSPC liposome.

Teske et al., (2011) studied the fatal intoxication due to brucine. These workers developed a sensitive method for identification and quantification of brucine using liquid chromatography-tandem mass spectrometry. The limits of detection and quantification were found to be 0.12 and 0.23ng/mL, respectively, based on a solid-phase extraction for human serum. In one case of lethal suicidal brucine monointoxication, brucine concentrations of 1.51 μg/mL, 1.69 μg/mL, 9.94 μg/mL, 16.4 μg/g, 0.99 μg/g, 0.75 μg/g, and 1.95 mg/g were determined in femoral blood, urine, bile collected from the gallbladder, liver tissue, cerebellum, cerebrum, and stomach contents, respectively.

Agrawal et al., (2011) studied the cytotoxic and antitumor effects of brucine on Ehrlich ascites tumor and human cancer cell line. These workers examined cell proliferation and viability using microculture tetrazolium tests (MTT). Antitumor activity was evaluated against Ehrlich ascites and solid tumor models. 15×10(6) EAC cells were implanted intraperitoneally (i.p., ascites tumor) and subcutaneous (s.c., solid tumor) in Swiss albino mice. Mice with established tumors received brucine i.p. at 12.5, 25, and 50mg/kg for 14days in ascites tumor and 50mg/kg in solid tumor for 30days.

2.8. Analytical review of strychnine and brucine

Zong and Che (1995) determined strychnine and brucine by capillary zone electrophoresis. These workers developed a method for quantitative estimation of strychnine and brucine in the extracts of Strychnos nux-vomica seeds by capillary zone electrophoresis (CZE). A 10mM phosphate buffer-MeOH (9:1) buffer solution with pH 2.5 was used and the calibration curve was linear in the range of 0.01-0.15 mg/ml.

De Saqui-Sannes et al., (1996) determined strychnine and crimidine in biological samples using a HPTLC method. In this method, fortified dog serum and stomach content samples were analyzed after extraction with chloroform and quantified by densitometry in the ultraviolet (UV) range (260 nm) using HPTLC silica gel 60 plates. The limit of detection was as low as 5 ng and the linearity was achieved over a range of 10-250 ng deposits for crimidine and 12.5-250 ng deposits for strychnine with simple or F_{254} plates.

Gu et al. (1997) determined strychnine and brucine in the seeds, root, stem and leaves of Strychnos nux-vomica by HPLC. The separation was performed on an analytical column ZY110 YNG-C_{18} using mobile phase KH_2PO_4 (0.01 mol.L-1)—MeOH (73:27) with pH 2.5 regulated by 10% H_3PO_4. The recoveries of strychnine and brucine were found to be 98.27% and 98.04%, respectively. The contents of strychnine in the seeds of Strychnos wallichiana and S. ignatii were 5.6% and 3.9%, respectively.

Jiang et al., (2002) determined strychnine and brucine in Semen Strychni and its processed products of Jiangxi method by HPLC. In this method, SiO_2 was used as the stationary phase, n-hexane-dichloromethane-methanol-ammonia (47.5:47.5:5:0.35) as the mobile phase, with detection wavelength of 254 nm.

Frédérich et al., (2003) performed quantitative analysis for the determination of strychnine and Brucine in Strychnos nux-vomica seeds and stems using (1) H-NMR (Q-NMR). In this method, no reference alkaloids were used for calibration curves, the quantification was performed on a crude extract, an overall profile of the preparation (including non alkaloid compounds) was directly obtained, and a very significant time-gain was achieved, in comparison to conventional HPLC methods.

Zhang et al., (2006) performed rapid separation of strychnine and brucine on a dynamically modified poly (dimethylsiloxane) (PDMS) microchip followed by electrochemical (EC) detection. The two analytes are well separated within 90 s in 70 mmol/L acetate buffer (pH 5.5) containing 0.01% (v/v) Brij35. The limits of detection were found to be 1.0 micromol/L for strychnine and 0.2 micromol/L for brucine at S/N = 3.

Xuexin et al., (2006) determined strychnine and brucine in Strychnos *nux-vomica* L. by nonaqueous capillary electrophoresis with photo diode-array detection. The separation of the two alkaloids was achieved within 10 min using a running buffer containing 25 mM Tris-boric acid, 60% methanol and 20% acetonitrile with acetic acid adjusting pH to 4.0.

Li et al., (2006) simultaneously determined strychnine and brucine in Strychnos nux-vomica L. and its preparation by nonaqueous capillary electrophoresis (NACE) without pretreatment for the first time. The separation was achieved with a fused-silica capillary column (50 cm x75 microm i.d.) and a running buffer containing 30 mM ammonium acetate, 1.0% acetic acid and 15% acetonitrile (ACN) in methanol medium. The method was linear over the range of 5-1000 mg/mL for both strychnine and brucine and recoveries were found to be 94.5 to 104%.

Dhalwal et al., (2007) developed and validated a TLC-densitometric method for the simultaneous quantitation of strychnine and brucine from Strychnos spp. and its formulations. The chromatographic separation was achieved on silica gel plate with toluene-ethyl acetate-diethyl amine-methanol (7:2:1:0.3 v/v) as the mobile phase. The method was linear over a concentration range of 160 to 480 ng/spot for strychnine and 80 to 480 ng/spot for brucine. Instrumental precision was found to be 0.54 and 0.78 (% CV), and repeatability of the method was 1.01 and 1.06 (% CV).

Pietsch et al., (2008) simultaneously determined thirteen plant alkaloids (aconitine, anabasine, atropine, brucine, colchicine, cotinine, cytisine, harmine, ibogaine, nicotine, scopolamine, strychnine, yohimbine) in a human specimen by solid-phase extraction (SPE) and reversed-phase high performance liquid chromatography (RP-HPLC) with photodiode array detection.

Qiu et al. (2008) simultaneously determined five toxic alkaloids: Brucine, Strychnine, Ephedrine, Aconitine and Colchicine, in blood and urine by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry in the multiple reaction monitoring (HPLC-ESI-MRM) mode. The linearity range for Brucine was 0.05-50.0 ng/mL, 0.1-50.0 ng/mL for Strychnine and Ephedrine, 0.01-10.0 ng/mL for Aconitine and Colchicine. The limits of quantification for Brucine, Strychnine, Ephedrine, Aconitine and Colchicine were found to be 0.03, 0.05, 0.20, 0.05, 0.01 ng/mL, respectively. The average extraction recoveries in urine ranged from 96.0 to 114.0% and in whole blood were 94.0 to 113.0%.

Xu et al., (2009) developed and validated a method for simultaneous determination of strychnine and brucine in rat plasma, using tacrine as the internal standard (IS) by liquid

chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). Chromatographic separation was carried out on a Waters C 18 column using a mobile phase of methanol-20mM ammonium formate-formic acid (32:68:0.68, v/v/v). Linearity was obtained over the concentration range of 0.5-500 ng/mL for strychnine and 0.1-100 ng/mL for brucine. The lower limit of quantification was 0.5 ng/mL and 0.1 ng/mL for strychnine and brucine, respectively.

Li and Jiang (2010) simultaneously determined strychnine and brucine in human urine by capillary electrophoresis (CE) coupled with field-amplified sample stacking (FASS). The calibration curves were linear over a range of 8.00-2.56 infinity 10(2) ng/mL (r = 0.9995) for strychnine and 10.0-3.20 x 10(2) ng/mL (r = 0.9999) for brucine. Extraction recoveries in urine were greater than 79.6 and 82.8% for strychnine and brucine, respectively, with an RSD of less than 4.9%. The detection limits (signal-to-noise ratio 3) for strychnine and brucine were 2.00 and 2.50 ng/mL, respectively.

Ganesan et al., (2010) simultaneously determined strychnine and brucine in herbal formulation by UV first order derivative spectrophotometry. The linear was over the range of 10-50 mg/mL for strychnine and brucine, respectively. The strychnine and brucine in herbal formulation were quantified using the first order derivative spectrum in which strychnine detected at 265.4 nm and brucine detected at 256.4 nm.

Chen et al., (2011) determined strychnine and brucine in rat tissues (kidney, liver, spleen, lung, heart, stomach, small intestine, brain and plasma) by HPLC with UV detection and studied the distribution of processed semen strychni. The LOQs were in the range of 0.039-0.050 μg/ml for different tissues or plasma samples. The extraction recoveries varied from 71.63 to 98.79% and

the linear range was 0.05-2 µg/ml with correlation coefficient of over 0.991.The highest level was observed in kidney, while the lowest level was found in brain.

Liu et al., (2011) developed an ultra-performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) method for the determination of strychnine and brucine in mice plasma. The method was validated over the range of 2.48-496.4 ng/ml for strychnine and 2.64-528 ng/ml for brucine, respectively. Intra- and inter-day accuracy ranged from 95.0% to 107.9% for strychnine, 93.4% to 103.3% for brucine, and the precisions were within 13.8%. The extraction recoveries of both the two alkaloids exceed 81.9%.

2.9. PHARMACOLOGICAL PROPERTIES OF PODOPHYLLOTOXIN

Pugh et al., (2001) investigated that podophyllotoxin lignans enhance IL-1beta but suppress TNF-alpha mRNA expression in LPS-treated monocytes. These workers examined the effect of podophyllotoxin and six analogs on nuclear factor kappa B (NF-kappa B) activation, and on interleukin-1 beta (IL-1beta) and tumor necrosis factor alpha (TNF-alpha) mRNA expression in human THP-1 monocytes.

Rothenborg-Jensen et al., (2001) examined that the linker length in podophyllotoxin-acridine conjugates determines potency in vivo and in vitro as well as specificity against MDR cell lines. These workers synthesized two podophyllotoxin-acridine conjugates-pACR6 and pACR8. These results exhibit that the spatial orientation of podophyllotoxin and acridine moieties in hybrid molecules determine target interaction as well as substrate specificity in active drug transport.

Tian et al., (2002) examined the antitumor and antioxidant activity of spin labeled derivatives of podophyllotoxin (GP-1) and congeners. The results showed that the anticancer activity of these compounds followed the order GP-1 >GP-1-OH> GP-1-H. It can be attributed to the influence of their partition coefficients and ionization constants to the compounds properties. It can be concluded that the oxidative state of nitroxide in compounds play a key role to the antioxidant activity.

Roulland et al., (2002) performed the hemi-synthesis and biological activity of new analogues of podophyllotoxin. Various four analogues of podophyllotoxin and epipodophyllotoxin were obtained via the formation of the corresponding 4-keto derivatives. Methyloximation of podophyllotoxone, followed by subsequent catalytic hydrogenation, gave stereoselective access

to 4-alpha-amino-4-deoxypodophyllotoxin and from there, to the corresponding acetamido and formamido derivatives.

Castro et al., (2003) performed the synthesis and cytotoxicity of podophyllotoxin analogues modified in the A ring. Several podophyllotoxin derivatives lacking the methylenedioxy group or with different functionalization of the A-ring of the cyclolignan skeleton have been prepared and evaluated for their cytotoxic activities on four neoplastic cell lines (P-388, A-549, HT-29 and MEL-28). Most of them maintained their cytotoxicity at the microM level.

Bala and Goel (2004) studied the radioprotective effect of podophyllotoxin in Saccharomyces cerevisiae. Proliferating yeast cells pretreated with podophyllotoxin (2.5-5.0 microg/mL) for > or =3 hours showed a higher surviving fraction after (60) Co-gamma-irradiation (200-600 Gy) than did the irradiated cells not pretreated with podophyllotoxin. The maximum increase (2.0 times) in surviving fraction was observed in cells treated with 2.5 microg/mL podophyllotoxin, 5 hours before (60) Co-gamma-irradiation (400 Gy).

Zhang et al., (2005) examined the apoptosis induced by one new podophyllotoxin glucoside in human carcinoma cells. 4-Demethyl-picropodophyllotoxin 7'-O-beta-D-glucopyranoside (4DPG), a new podophyllotoxin glucoside, was isolated from the rhizomes of Sinopodophyllum emodi (Wall.) Among the target cells (HeLa, A2 and SH-SY5Y), the cytotoxic effects of 4DPG showed dose- and time-dependency.

Lin et al., (2006) examined that deoxypodophyllotoxin (DPT) inhibits eosinophil recruitment into the airway and Th2 cytokine expression in an OVA-induced lung inflammation. DPT (1.0 to 5 mg/kg) was given orally to ovalbumin (OVA)/alum-induced asthmatic mice. DPT reduced the

number of infiltrated eosinophils in bronchoalveolar lavage (BAL) fluid in a dose-dependent manner.

Imperio et al., (2007) studied that the replacement of the lactone moiety on podophyllotoxin and steganacin analogues with a 1,5-disubstituted 1,2,3-triazole via ruthenium-catalyzed click chemistry. Theses workers performed the ruthenium-catalyzed [3+2] azide-alkyne cycloaddition, a click-chemistry reaction, to replace the lactone ring with a 1,5-disubstituted triazole in few synthetic steps. The compounds were cytotoxic, although to a lesser degree compared to podophyllotoxin, while retaining antitubulin activity.

Miao et al., (2008) investigated that Seleno-podophyllotoxin derivatives induce hepatoma SMMC-7721 cell apoptosis through Bax pathway. These workers evaluated the anti-tumor activity of a new isolated derivative of podophyllotoxin, 4'-demethyl-4-dehydroxy-4-seleno-phenyl-beta-peltatin-epipodophyllotoxin (CPZ) and found that CPZ can suppress the proliferation of human hepatoma SMMC-7721 cells in a dose- and time-dependent manner. Rh123 label testing revealed that the mitochondrial membrane potential had been decreased by CPZ treatment.

Chen et al., (2009) examined L1 EPO, a novel podophyllotoxin derivative overcomes P-glycoprotein-mediated multidrug resistance in K562/A02 cell line. These workers investigated the inhibitory effects of L1EPO (after its synthesis) on P-glycoprotein (P-gp)-mediated MDR in K562/A02 and KBv200 cell lines, which expressed high levels of P-gp. It was found that the K562/A02 cell line was rendered resistant toward Adriamycin but not towards L1EPO when compared with the parental cells.

Chen et al., (2010) investigated a novel podophyllotoxin derivative (YB-1EPN) which induces apoptosis and down-regulates express of P-glycoprotein in multidrug resistance cell line KBV200. These workers synthesized a new potent anti-tumor podophyllotoxin derivative, YB-1EPN. It was also observed that the KBV200 cell line and K562/A02 cell line were rendered resistant towards VP-16 but not towards YB-1EPN.

Labruere et al., (2010) performed the design, synthesis, and biological evaluation of the first podophyllotoxin analogues as potential vascular-disrupting agents. These workers designed and synthesized two novel series of azapodophyllotoxin analogues as potential antivascular agents. These workers identified several new compounds with inhibitory activity toward tubulin polymerization similar to that of CA-4 and colchicine, while displaying low cytotoxic activity against normal and/or cancer cells.

Kamal et al., (2010) performed the synthesis of 4β-N-polyaromatic substituted podophyllotoxins: DNA topoisomerase inhibition, anticancer and apoptosis-inducing activities. A new class of 4β-N-polyaromatic substituted podophyllotoxin congeners have been synthesized and evaluated for their DNA topoisomerase-II (topo-II) inhibition as well as anticancer potential in some human cancer cell lines.

Guo et al., (2011) examined the synthesis and antitumor activity of novel podophyllotoxin derivatives against multidrug-resistant cancer cells. Seven novel 4β-N-substituted podophyllotoxin derivatives with indole rings were prepared and evaluated for cytotoxicity against human cancer cell lines HeLa, KB, KBV, K562, and K562/AO2. Most of them demonstrated improved antitumor activity and weak multidrug resistance compared to the drugs currently available.

Kamal et al., (2011) synthesized 4β-carbamoyl epipodophyllotoxins which acts as potential antitumour agents. A series of new 4β-carbamoyl epipodophyllotoxin analogues have been synthesized and evaluated for their anticancer activity against eleven cancer cell lines including Zr-75-1, MCF7, KB, Gurav, DWD, Colo 205, A-549, Hop62, PC3, SiHa and A-2780. Most of the compounds exhibited better growth-inhibition activities against tested cell lines than that of etoposide.

Magedov et al., (2011) examined that anticancer properties of an important drug lead podophyllotoxin can be efficiently mimicked by diverse heterocyclic scaffolds accessible via one-step synthesis. The most potent compounds were found in the dihydropyridopyrazole, dihydropyridonaphthalene, dihydropyridoindole, and dihydropyridopyrimidine scaffold series. Biochemical mechanistic studies performed with dihydropyridopyrazole compounds showed that these heterocycles inhibit in vitro tubulin polymerization and disrupt the formation of mitotic spindles in dividing cells at low nanomolar concentrations, in a manner similar to podophyllotoxin itself.

2.10. PHARMACOLOGICAL PROPERTIES OF ETOPOSIDE

Chen et al., (2001) studied the bioavailability and pharmacokinetic features of etoposide in 12 relapsed B-lineage acute lymphoblastic leukemia (ALL) patients after both intravenous (i.v.) infusion and oral administration. Following a 1 hour i.v. infusion of 50 mg/m2 etoposide, the elimination half-life ranged from 49.8 min to 509.4 min (mean +/- SD = 218.6 +/- 134.7 min), the MRT ranged from 71.8 to 734.9 min (mean +/- SD = 315.4 +/- 194.3 min) and the systemic clearance of etoposide ranged from 15.7 to 38.0 ml/min/m2 (mean +/- SD = 24.1 +/- 7.0 ml/min/m2).

Custodio et al., (2001) studied the mitochondrial permeability transition induced by the anticancer drug etoposide. These workers evaluated the interference of VP-16 with MPT in vitro, which is characterized by the Ca (2+)-dependent depolarization of Delta Psi, the release of matrix Ca (2+) and by extensive swelling of mitochondria. Incubation of rat liver mitochondria with VP-16 results in a concentration-dependent induction of MPT, evidenced by an increased sensitivity to Ca (2+)-induced swelling, depolarization of Delta Psi, Ca (2+) release by mitochondria and stimulation of state 4 oxygen consumption.

Kagan et al., (2001) studied the pro-oxidant and antioxidant mechanisms of etoposide in HL-60 cells: role of myeloperoxidase. Etoposide acted as an effective radical scavenger and antioxidant protector of phosphatidylethanolamine, phosphatidylcholine, and other intracellular phospholipids against H_2O_2-induced oxidation in HL-60 cells with constitutively high MPO activity and in HL-60 cells depleted of MPO by an inhibitor of heme synthesis, succinyl acetone.

Watanabe et al. (2002) investigated the chemotherapeutic targeting of etoposide to regions of the brain on the basis of polyamine level. The effects of etoposide on body weight, regional

weights, and the concentrations of putrescine, spermidine and spermine in the cerebellum, hippocampus, corpus striatum, cortex, the combined regions of the thalamus and hypothalamus and the diencephalon of the brain were examined in rats.

Bar Sela et al., (2002) examined etoposide, doxorubicin and cisplatin alternating with 5-fluorouracil, doxorubicin and high-dose methotrexate in patients with advanced adenocarcinoma of the stomach or the gastroesophageal junction. These workers took a phase II trial to evaluate EAP (etoposide, doxorubicin, cisplatin) and FAMTX (high-dose methotrexate) in patients with advanced adenocarcinoma of the stomach or gastroesophageal junction. Of the 56 patients treated, an objective response was observed in 34%, including complete response in 7%. Median response duration was 8 months and median survival for the entire group was 9 months.

Nesina et al., (2003) asudied the effects of etoposide and fludarabine in subtoxic doses on karyotype of human malignant lymphoid Namalwa cells. These workers studied changes in the karyotype of transplanted Namalwa cells induced by DNA-damaging antitumor preparationsetoposide and fludarabine in subtoxic doses. The relative number of cells containing increased number of chromosomes and the incidence of chromatid aberrations with primary damage to chromosomes 2, 5, 11, 16, and 17 increased.

Kimura et al., (2003) performed intermittent oral hormonal chemotherapy using estramustine phosphate and etoposide for the treatment of hormone-refractory prostate cancer. Seventeen patients were given lower dose and intermittent oral administration of estramustine phosphate (6 mg/kg/day) andetoposide (30 mg/m2/day) for 7 days. None of the patients showed edema, deep venous thrombosis, thrombocytopenia, anemia or myocardial infarction.

Blanco et al., (2004) studied that etoposide induces chimeric Mll gene fusions. These workers developed a long-distance inverse PCR DNA-based assay for chimeric Mll fusions in mouse embryonic stem cells. It was found that Mll fusions at a higher frequency following 100 microM etoposide for 8 h (16x10(-6) cell (-1)) than in no-drug controls (1.0x10(-6) cell(-1), P=0.0002) or after treatment with a comparably cytotoxic exposure to the antimicrotubule drug vincristine (1.0x10(-6) cell(-1), P=0.0047).

Zhuo et al., (2004) studied the kinetics and regulation of cytochrome P450-mediated etoposide metabolism. Kinetic analysis of catechol formation by recombinant P450s was determined using liquid chromatography/selected reaction monitoring/mass spectrometry. CYP3A4 was found to play a major role in etoposidemetabolism (K (m) = 77.7 +/- 27.8 microM; V(max) = 314 +/- 84 pmol of catechol/min/nmol of P450). Etoposide (40, 5, 1, and 0.25 microM) caused a slight increase in CYP3A4 mRNA in three of five batches of hepatocytes but did not result in proportionately increased CYP3A4 protein levels.

Patlolla and Vobalaboina (2005) studied the pharmacokinetics and tissue distribution of etoposide delivered in parenteral emulsion. The particle size distribution with polydispersity indices, zeta potential, entrapment efficacy, and assay of EPE were found to be 218.7 +/- 4.7 (0.14 +/- 0.0) nm, -53.5 +/- 0.2 mV, 75 +/- 2.1%, and 0.85 +/- 0.1 mg/mL, respectively. The EPE showed high AUC (0-alpha), MRT (mean residence time), and lower clearance than that of ETP.

Park and Kim (2005) studied the release of cytochrome c from isolated mitochondria by etoposide. These authors showed that etoposide can induce the similar degree of cell death in p53-deficient HCT 116 cells, whereas 5-FU-mediated cell death is strongly dependent on the existence of functional p53 in HCT 116 cells.

Merchetti et al., (2006) studied that etoposide induces chromosomal abnormalities in mouse spermatocytes and stem cell spermatogonia. Chromosomal aberrations (partial duplications and deletions) and whole chromosomal aneuploidies were detected in sperm of mice treated with a clinical dose of ET. ET treatment resulted in major increases in the frequencies of sperm-carrying chromosomal aberrations in both meiotic pachytene (27- to 578-fold) and spermatogonial stem-cells (8- to 16-fold), but aneuploid sperm were induced only after treatment of meiotic cells (27-fold) with no persistent effects in stem cells.

Lamprecht and Benoit (2006) investigated that etoposide nanocarriers suppress glioma cell growth by intracellular drug delivery and simultaneous P-glycoprotein inhibition. In cell culture, an internalization of LNC was observed in all glioma cell types. Etoposide LNC showed a generally higher efficiency than the drug solution while blank LNC were found to be less inhibitory than the pure drug at equivalent concentrations (IC50: C_6: etoposide: 25.2 microM; LNC: 2.6-8.9 microM, F98: etoposide: 46.5 microM; LNC: 1.4-14.7 microM, 9L: etoposide: 58.2 microM; LNC: 4.4-12.7 microM).

Xian et al., (2007) studied the effects of etoposide and cyclophosphamide acute chemotherapy on growth plate and metaphyseal bone in rats. These authors investigated the effects of acute chemotherapy with topoisomerase inhibitoretoposide (Eto, 80 mg/kg), alkylating agent cyclophosphamide (Cyc, 240 mg/kg) or their combination (Cyc 120 mg/kg + Eto 50 mg/kg) on structural and cellular changes in the growth plate cartilage and metaphyseal bone, two important regions responsible for bone growth and bone mass accumulation.

Seo et al., (2007) investigated a case of therapy-related acute monocytic leukemia following low-dose of etoposide treatment for hemophagocytic lymphohistiocytosis. These workers reported a

case of therapy-related acute myeloid leukemia after low-dosed topoisomerase II inhibitor (etoposide) treatment for hemophagocytic lymphohistiocytosis (HLH).

Snehalatha et al., (2008) studied etoposide loaded PLGA and PCL nanoparticles II, their biodistribution and pharmacokinetics after radiolabeling with Tc-99m. A higher proportion of nanoparticles compared with etoposide was observed in different organs of mice. Scintigraphic images of rabbits concluded that the radioactivity shown by formulations is significantly higher after 4 and 24 h, as compared with etoposide administered in rabbits.

Remichkova et al., (2008) investigated that etoposide attenuates zymosan-induced shock in mice. In the present study, these authors investigated whether the ability of etoposide to diminish macrophage number would have an impact on the course of zymosan-induced shock. The drug significantly reduced the mortality and decreased the organ toxicity in Balb/c mice. Simultaneously, an inhibition of TNF-alpha production by alveolar and peritoneal macrophages was observed.

Daw et al., (2009) studied the renal function after ifosfamide, carboplatin and etoposide (ICE) chemotherapy, nephrectomy and radiotherapy in children with Wilms tumour. These authors evaluated tumour response and renal function in 12 newly diagnosed children with high-risk Wilms tumour receiving ifosfamide, carboplatin and etoposide (ICE) chemotherapy. Mean GFR (glomerular filtration rate) declined by 7% after 2 cycles of ICE and by 38% after nephrectomy; the mean carboplatin dose was reduced 32% after nephrectomy.

Sistla et al., (2009) studied the pharmacokinetics and tissue distribution of liposomal etoposide in rats. The pharmacokinetics and distribution of the commercial formulation (ETPI) and LE were

compared in rats. The pharmacokinetic profiles were biphasic and similar in the initial phase (C (max), Vd, and t (1/2alpha)). However, LE showed a 60% increase in AUC with a 35% decrease in clearance ($p < 0.05$). This decreased clearance resulted in a 70% increase in the MRT of etoposide.

Verma and Hansch (2010) performed a QSAR study on the cytotoxicity of podophyllotoxin analogues against various cancer cell lines. In this method, the cytotoxicity data of two series of podophyllotoxin derivatives against four different cancer cell lines was used to develop 4 QSAR models.. The developed QSAR models showed a good correlative and predictive abilities having r (2) = 0.960 to 0.836 and q (2) = 0.911 to 0.705. On the basis of QSAR 1, two compounds (10-10 and 10-11) are suggested as potential synthetic targets.

Nam et al., (2010) investgated that etoposide induces G2/M arrest and apoptosis in neural progenitor cells via DNA damage and an ATM/p53-related pathway. These workers injected 4 mg/kg of VP-16 into pregnant mice on day 12 of gestation, and the fetuses were investigated for the cell cycle checkpoint and mechanism of apoptosis. VP-16-induced S-phase accumulation was brought about by the acceleration of G1/S transition rather than by the inhibition of S-phase progression.

Meley et al., (2010) examined that p53-mediated delayed NF-κB activity enhances etoposide-induced cell death in medulloblastoma. These authors demonstrated that the chemotherapeutic drug etoposide induces a p53- and caspase-dependent cell death. These workers also observed an additional caspase-independent cell death mechanism involving delayed nuclear factor κB (NF-κB) activity.

Chrzanowska et al., (2011) studied the pharmacokinetics of high-dose etoposide administered in combination with fractionated total-body irradiation as conditioning for allogeneic hematopoietic stem cell transplantation in children with acute lymphoblastic leukemia. VP-16 plasma concentrations were determined using validated HPLC method. The median value of VP-16 C (max) measured at the end of infusion was 188.0 μg/mL (range 148.0-407.0 μg/mL). Out of 21 studied children, VP-16 was still detectable in 17 patients 72 h and in eight patients 96 h after the end of infusion.

Jacob et al., (2011) investgated that etoposide quinone is a redox-dependent topoisomerase II poison. These workers examined the ability of etoposide quinone to poison human topoisomerase IIα in the absence of reducing agents. Under these conditions, etoposide quinone was ~5-fold more active than etoposide at inducing enzyme-mediated DNA cleavage Unlike etoposide, the quinone metabolite did not require ATP for maximal activity and induced a high ratio of double-stranded DNA breaks.

2.11. Analytical review of podophyllotoxin and etoposide

Chen and Uckun (2000) performed a highly sensitive liquid chromatography-electrospray mass spectrometry (LC-MS) method for the determination of etoposide levels in human serum and plasma. Etoposide was separated using Lichospher 100 RP-18 (5 microm) column (250 mmx4 mm) with the mobile phase of acetonitrile-water containing 0.1% acetic acid (45/55, v/v) at flow-rate of 0.5 ml/min. Good linearity (r>0.9965) was observed between concentrations of 0.0125-5 microM in 200 microl serum and 0.01-10 microM in 100 microl plasma.

Zhou et al., (2001) determined etoposide levels in human plasma, total and non-protein bound concentration, and in leukemic cells by high-performanceliquid chromatography with electrochemical detection. The precision for between-runs (n=6) was 7.0, 4.9, and 9.5%, the accuracy was 3.7, 7.1 and 6.3%, and within-runs precision (n=6) was 3.9, 2.9 and 5.1% for total plasma, non-protein bound plasma fraction and leukemic cells, respectively.

Shirazi et al., (2001) developed a rapid reversed phase high performance liquid chromatographic method for determination of etoposide (VP-16) in human plasma. The mobile phase consisted of methanol: water (45:55 v/v) with phenacetin used as an internal standard. The extraction method showed a recovery of 91.5+/-3% for etoposide. The limit of detection of etoposide in plasma is 20ng/ml and the limit of quantitation is 40ng/ml.

Pang et al., (2001) simultaneously determined etoposide and its catechol metabolite in the plasma of pediatric patients by liquid chromatography/tandem mass spectrometry. Liquid chromatography was performed on a YMC ODS-AQ column. The limits of detection were 200 ng ml (-1) etoposide and 10 ng ml (-1) catechol metabolite in human plasma and 25 ng ml(-1) etoposide and 2.5 ng ml (-1) catechol metabolite in protein-free plasma, respectively.

Acceptable precision and accuracy were obtained for concentrations in the calibration curve ranges 0.2--100 microg ml (-1) etoposide and 10--5000 ng ml (-1) catechol metabolite in human plasma.

Kato et al., (2003) determined etoposide serum concentrations in small pediatric samples by an improved method of reversed-phase high-performance liquid chromatography. The efficiency of extraction from serum was 85.7 +/- 7.7% for etoposide and 81.1 +/- 8.4% for diphenylhydantoin, the internal standard. The serum concentrations of etoposide were measured in 0.2-ml serum samples. The lower limit of detection was 50 ng/ml. The linear quantitation range foretoposide was 0.05-50 microg/ml.

Fahmy et al., (2004) determined some co-administered anticancer drugs in pharmaceutical preparations and in spiked human plasma by high performance liquid chromatography (HPLC). These authors developed two HPLC methods for the simultaneous determination of doxorubicin hydrochloride (DOX) and 5-fluorouracil (5-FU), combination I, and of cytarabine (CYT) and etoposide (ETO), combination II, as co-administered drugs. In both combinations, a [250 mm x 4.6 mm C-18 column is used.

Mishra et al., (2005) determined Podophyllotoxin in *Podophyllum hexandrum* by reverse phase high performance thin layer chromatography (RP-HPTLC). The assay employed RP-18 F_{254} TLC plates (chromatoplates) as the stationary phase. The solvent system consisted of acetonitrile–water (4 : 6, v/v) and gave better resolution, with well separated, compact spots for podophyllotoxin (Rf value 0.41 ± 0.02).

Lee et al., (2008) studied the characterization of in vitro metabolites of deoxypodophyllotoxin in human and rat liver microsomes using liquid chromatography/tandem mass spectrometry. The in vitro metabolism of deoxypodophyllotoxin (DPT), a medicinal herbal product isolated from Anthriscus sylvestris (Apiaceae), was investigated in rats and human microsomes and human recombinant cDNA-expressed CYPs. Reasonable structures of the seven metabolites of DPT could be proposed, based on the electrospray tandem mass spectra.

Zhao et al., (2009) performed HPLC-DAD and HPLC-ESI-MS separation, determination and identification of the spin-labeled diastereoisomers of podophyllotoxin. In the HPLC-ESI/MS spectra, each pair of diastereoisomers of the spin-labeled derivatives in the mixture was directly confirmed and identified by [M+H](+) ions and ion ratios of relative abundance of [M-ROH+H](+) (ion 397) to [M+H](+). When the [M-ROH+H](+) ions (at m/z 397) were selected as the precursor ions to perform the MS/MS product ion scan. The product ions at m/z 313, 282, and 229 were the common diagnostic ions.

Krogh-Madsen et al., (2010) simultaneously determined cytosine arabinoside, daunorubicin and etoposide in human plasma. The assay was used for the simultaneous measurement of cytosine arabinoside, daunorubicin and etoposide with linearity in the ranges of 13-1500 ng/mL, 15-1000 ng/mL and 52.5-3500 ng/mL, respectively. The overall precision (% relative standard deviation) was within 0.2-13.5% and the recovery ranged between 86.1% and 110.1% for the three drugs at all concentrations tested.

Nussbaumer et al., (2010) performed a liquid chromatography separation with electrospray ionisation and tandem mass spectrometry for the simultaneous quantification of ten commonly handled cytotoxic drugs in a hospital pharmacy by a LC-ESI-MS/MS method. The analytical

method was validated to determine the limit of quantification (LOQ) and quantitative performance: lowest LOQs were between 0.25 and 2 ng mL(-1) for the ten investigated cytotoxic drugs; trueness values (i.e. recovery) were between 85% and 110%, and relative standard deviations for both repeatability and intermediate precision were always inferior to 15%.

Ronguist-Nii et al., (2011) determined picropodophyllin (AXL 1717) and its isomer podophyllotoxin in human serum samples with electrospray ionization of hexylamine adducts by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The chromatography system was a C18, column with gradient elution (mobile phase A: 2.5 mM hexylamine and 5 mM formic acid in Milli-Q water and mobile phase B: methanol). The limit of quantification (LOQ) was 0.01 μmol/L for picropodophyllin and podophyllotoxin. The limit of detection (LOD) was estimated below 0.001 μmol/L for picropodophyllin and podophyllotoxin.

Yuan et al., (2011) studied development and characterization of molecularly imprinted polymers for the selective enrichment of podophyllotoxin from traditional Chinese medicines. The results of the equilibrium rebinding experiments and the competitive adsorption experiments showed that these imprinted polymers exhibited good adsorption ability for the PPT. The regression equation was $y=5.873\times10^{6}x+17075.659$ with the correlation coefficient of 0.9994 in the concentration range of 0.005-0.4 mg mL^{-1}. The limits of detection were 0.12-0.18 μg mL^{-1} and their recoveries were in the range of 89.5-91.1% with all RSDs lower than 3.7.