PHARMACOGNOSTICAL AND PHYTOCHEMICAL EVALUATION

Quality control standardization of herbal drugs is an imperative step in correct identification and authentication of the plant material. Plants have been an integral part of pharmacotherapy throughout the history of mankind, and they have served as an invaluable source for the discovery of bioactive molecules since the very beginning of rational drug discovery in the 19th century (Kinghorn et al., 2011). Hence, it is very essential to obtain a proper quality control profile for various medicinal plant used in traditional system of medicine. Prime step of all, the starting materials need to be correctly authenticated and should be freed from adulterants and contaminants. During plant growth, many factors like harvest season and time, developmental stage, temperature, and humidity have a strong impact on plant metabolite production. Also, postharvest processing steps which include drying and storage can significantly alter the phytochemical composition of herbal material. As the production of many phytopharmaceuticals includes an extraction step, extraction conditions and solvents need to be optimized in order to enrich the bioactive constituents of the extracts. The quality of finished preparations needs to be determined either on the basis of marker constituents or on the basis of analytical fingerprints. Thus, all production stages should be accompanied by appropriate quality assessment measures (Pferschy-Wenzig and Bauer 2015) .This may be helpful in minimizing the adulteration which occurs mainly due to improper knowledge regarding the varied geographical conditions, associated problems of different vernacular names, its morphology and microscopy. It is also said that correct identification and proper quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which contributes to its safety and efficacy (Nagani et al., 2011). Organoleptic analysis revealed that fruit is a thick berry bearing numerous densely distributed white spots. It is well known that the microscopical examination of crude drug aims at

determination of the chemical nature of the cell wall along with the determination of the form and chemical nature of the cell contents. (Ahire et al., 2006)

Thus, it determines the size, shape and relative structure of different cells and tissue in a plant drug. Microscopic analysis revealed that fruit has fleshy thick pericarp which is differentiated into epicarp and thick mesocarp, fruit is developed from pentacarpellary syncarpous ovary, and the mature fruit consists of wide radiating carpel chambers with one or two seeds attached on axile placentum. It is well established that the moisture is an inevitable component of crude drug, which must be eliminated as far as practicable (Cho 2005).The extractive value determines the amount of active constituents in a given amount of medicinal plant material when extracted with the solvents. The extraction of any crude drug with a particular solvent yields a solution containing different phyto-constituents. The composition of these phyto-constituents in that particular solvent depends upon the nature of the drug and solvent used. Further, it is well established that the ash value is used to determine the quality and purity of drug (Cho 2005).The ash of any organic material is composed of ash of their non-volatile inorganic components. Further, the controlled incineration of crude drug resulted in an ash residue consisting of an inorganic material (metallic salts and silica). This value varies within fairly wide limits and is therefore an important parameter for purpose of evaluation of crude drug. Thus on the basis of physiochemical constants various parameters were quantified which includes the amount of ash values, extractive value, foreign matter, loss on drying, swelling index, foaming index, haemolytic index etc. Furthermore, pesticide residue and heavy metals content were also assessed in the fruits of *P. pashia* However, the values estimated were found to be within the limits as prescribe by the WHO guidelines (WHO 2002). Preliminary phytochemical screening revealed the presence of alkaloids, saponins, triterpenes, tannins, flavonoids, carbohydrates and sterols. Total phenolics and flavonoid analysis

revealed that fruits of *P. pashia* are rich in phenolics and flavonoids. Phenolics are well established compounds for biochemical activities such as antioxidant, antimutagenic, anticarcinogenic, as well as have ability to modify the gene expression.(Nakamura 2003) Moreover it has been reported that, certain phenols have been identified as potential neuroprotective compounds, aiming to one of the greater challenges in drug discovery today(Tapiero et al., 2002) Flavonoids in addition possess multifaceted beneficial bioactive qualities for health, such as antiallergic, anticancer, and anti-inflammatory activities, lowering blood pressure and plasma lipids and conferring cardioprotective and neuroprotective activities (Diaz et al., 2012). Experimental evidences clearly demonstrated that flavonoids exerts antiepileptic activity by modulating the $GABA_A$-Cl^- channel complex, due to their structural similarities with benzodiazepines (Wasowski and Marder 2012). Flavonoids isolated from plants are used as sedatives and tranquilizers, since they possess remarkable activity for ($GABA_A$) receptors and some of these possess pharmacological profile compatible with the above (Viola 1994). Our earlier research established the richness of EPP in polyohenolics (Sharma et al., 2017). Polyphenolic analysis of EPP showed the presence of several polyphenols such as rutin, myricetin, chlorogenic acid, syringic acid, chrysin etc. Interestingly, chrysin was found to be one of the major polyphenol (26.06±0.21mg/gm) in the abovementioned extract. It has been well established that polyphenols can easily penetrate the blood–brain barrier and exert a neuroprotective effect (Schaffer and Halliwell 2012) Hence, chrysin, which was the major polyphenol in EPP was isolated to identify its plausible role in the anticonvulsant activity of EPP. Moreover it was selected as the marker compound for standardization of extract, quantification results revealed that the content of chrysin in the crude ethanol extracts of *Pyrus pashia* was found to be 1.73% % w/w.

PHARMACOLOGICAL EVALUATION

In the present study, we for the first time report the anticonvulsant activity of EPP (100, 200, and 400 mg/kg) against an acute model of MES- and PTZ-induced convulsions in experimental animals. Furthermore, chrysin (2.5, 5, and 10 mg/kg), isolated from EPP, exhibited significant anticonvulsant activity against an acute model of PTZ-induced convulsions in rats indicating the fact that chrysin is perhaps one of the anticonvulsant agents of EPP. In addition, EPP and chrysin did not exhibit sedative-like behavior in the experimental rodents suggesting its safe profile. These observations emphasize the fact that EPP could be considered as an alternative therapeutic option in the management of epilepsy. In the present study, EPP did not exhibit any sign and symptoms of toxicity in the toxicological studies. All the selected doses of EPP exhibited anticonvulsant activity against MES-induced seizures in experimental animals. Interestingly, the two higher doses of EPP exerted a significant increased anticonvulsant activity than the lower dose of EPP in the MES model of epilepsy. Moreover, EPP (200 and 400 mg/kg) and the standard drug exhibited a similar therapeutic profile on these animals. It is well accepted that MES induces generalized acute tonic–clonic seizures and antiepileptic drugs such as phenytoin, the standard drug in the present study, and attenuates the progress of seizure through blocking the voltage-dependent sodium channels (White et al., 1997). It has also been suggested that the activity of monoaminergic system plays a significant role in the pathogenesis of MES-induced convulsions (Ritz and George 1997).Hence, it can be assumed that EPP may exert anticonvulsant effect against acute seizures of generalized tonic clonic type through blockade of the voltage dependent sodium channel and modulatory effect on monoaminergic system which has to be validated with future studies. On the contrary chrysin failed to protect the animals against MES induced convulsions. Indicating the fact that chrysin is not having activity against sodium channels.

PTZ produces generalized absence seizures through antagonizing GABA receptor in an uncompetitive manner and picrotoxin sensitive site of GABAA receptor complex, and stimulation of N-methyl D-aspartate (NMDA) receptors. EPP (200 and 400 mg/kg) and chrysin (5 and 10mg/kg) showed better anticonvulsant effect than its low dose level against PTZ-induced seizures in experimental animals. EPP (200 and 400 mg/kg), chrysin (5 and 10mg/kg) and the standard drug, DZ, exhibited similar therapeutic profile against PTZ-induced seizures in rodents. It is well known that DZ is famous for its GABA facilitator property (Macdonald and Kelly 1995) and thus it can be assumed that EPP could have multiple actions on GABAA and NMDA receptor activity in addition to voltage-dependent sodium channel blocking and monoaminergic modulatory activity.

Chrysin (2.5, 5, and 10 mg/kg) attenuated acute generalized tonic–clonic seizures induced by PTZ in experimental rodents in the present study. Furthermore, the electrophysiological study revealed that chrysin and EPP exhibited a specific pattern of EEG fingerprint in cortical regions of PTZ challenged animals. Chrysin at the low dose level attenuated acutely induced an increase in the frequency of all waves in cortical regions of PTZ challenged animals. It is interesting to note that both the higher doses of chrysin and extract further attenuated an acutely induced an increase in the frequency of all waves in cortical regions of PTZ challenged animals which was similar to that of the standard drug. These observations indicate a fact that there is a ceiling effect in the anticonvulsant activity of chrysin. Moreover, chrysin and EPP may be effective on sensorimotor cortical areas of the rat brain, which has to be supported with future studies.

Sudden and recurrent electrical discharges cause an increase in the concentration of reactive oxygen species (ROS) including superoxide anions, hydroxyl radicals, and hydrogen peroxide in the brain. These ROS are the predisposing factors for oxidative stress and concurrent neuronal

damage in the brain. Polyphenols are well-recognized potent antioxidants that protect against ROS-induced neuronal damage and thus neurological diseases (Rodriguez et al., 2012) Further investigations in the present study revealed that EPP (100, 200 and 400), chrysin (2.5, 5, and 10 mg/kg) significantly attenuated PTZ-induced changes in all the oxidative stress biomarkers in the selected rat brain regions. Therefore, chrysin and EPP could ameliorate the convulsion-induced oxidative stress in the rat brain.

It has been well reported that histidine residue containing α1 subunit of $GABA_A$ receptor is sensitive to benzodiazepine such as DZ and its activation promotes sedation in mammals (Rudolph and Mohler 2004). Though chrysin (5 and 10 mg/kg) and EPP (200 and 400 mg/kg) exhibited similar therapeutic profile to that of DZ in the PTZ-induced animal model of epilepsy, however, it did not cause sedation in the normal animals. Therefore, it can be assumed that chrysin in EPP may exert anticonvulsant activity perhaps through binding activity at α2/α3 subunits of GABAA receptor as these subunits do not possess sedative-like effects in mammals.

Results of the present study provide evidence that chrysin possesses anticonvulsant activity in the pharmacologically validated experimental animal models used. Pentylenetetrazole (PTZ) test is the most frequently used acute chemical experimental model employed in the search for new antiepileptic drugs (AEDs) (Loscher, 2011). PTZ blocks GABA-mediated Cl– influx through an allosteric interaction in the Cl– channel, thus leading to induction of convulsions in animals (Velíšek, 2006). The GABAergic system is implicated in epilepsy since enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsion respectively (Meldrum, 1981; Gale, 1992; Quintans-Júnior *et al.*, 2008). Defects in GABA neurotransmission are linked to epilepsy in both experimental animal models and human syndromes (Velíšek, 2006). Drugs such as benzodiazepines, phenobarbitone, valproate and felbamate that enhance $GABA_A$

receptor-mediated inhibitory neurotransmission can also block PTZ-induced clonic seizures (Macdonald and Kelly, 1995). Ability of an agent to prevent or delay the onset of clonic and tonic-clonic convulsions induced by PTZ in animals is an indication of anticonvulsant activity (Amabeoku and Chikuni, 1993). In this study, acute administration of chrysin and the benzodiazepine diazepam, exhibited anticonvulsant activity against PTZ- induced seizures by significantly delaying the occurrence of clonic seizures. In addition chrysin and diazepam exhibited 100% of mice protection. This potent effect of diazepam as evident in the PTZ-induced convulsions agrees with its enhancing effects in GABAergic neurotransmission (Patil et al., 2011). Therefore, the blocking effect of chrysin in the PTZ-seizure model suggests its anticonvulsant action may probably be due to its interference with GABAergic mechanism(s). It has also been suggested that PTZ-induced clonic seizures model myoclonic seizures (Tchekalarova, 2009). Thus, chrysin could protect against myoclonic seizures. The convulsant action of picrotoxin is via blockade of the GABAA receptor-linked chloride ion channel, which normally opens to allow increased chloride ion conductance following the activation of $GABA_A$ receptors by GABA (Velíšek, 2006). Data from this study shows that chrysin and diazepam exhibited anticonvulsant activity against picrotoxin-induced seizures by significantly and dose-dependently delaying the occurrence as well as decreasing the frequency and duration of clonic seizures. It is probable that chrysin attenuated picrotoxin-induced convulsions by enhancing GABA neurotransmission. This further supports the hypothesis that chrysin may be affecting GABAergic mechanism(s) to exert its anticonvulsant activity. Isoniazid induces convulsions by inhibiting GABA synthesis (Costa *et al.*, 1975). It inhibits glutamic acid decarboxylase (GAD) activity (enzyme involved in GABA synthesis), resulting in decreased levels of GABA (Raygude et al., 2012). Similar to diazepam, chrysin significantly delayed convulsions (clonic and tonic) and reduced mortality indicating

anticonvulsant effect against INH-induced convulsions. Action against INH-induced convulsions could therefore indicate increased GABA synthesis by chrysin. This further confirms the possible contribution of the GABAergic system in the anticonvulsant activity of the chrysin. To further confirm the possible involvement of GABAergic system in the anticonvulsant activity of PME, flumazenil, a specific antagonist of the benzodiazepine site in the $GABA_A$ BZD receptor complex (Brogden and Goa, 1991), was used. Pretreatment with flumazenil antagonised the anticonvulsant effect of PME suggesting involvement of the $GABA_A$ receptor complex. Activation of glycine receptors results in an influx of chloride ions into the neuron, which is then hyperpolarized and inhibited. Strychnine acts as a selective competitive antagonist that blocks the inhibitory effect of chloride channel associated with glycine at all glycine receptors (Curtis et al., 1971). Thus, the observed protection of diazepam or the chrysin in the strychnine-induced seizure test is presumably mediated through the glycinergic pathway. Pretreatment of carbamazepine before administration of 4-AP significantly increased the latency to seizures and reduced the incidence of mortality. However, chrysin neither increased latency nor reduced the mortality in experimental animals. Action of 4-AP occurs through a K+-channel blockade at the presynaptic neuronal level (Thesleff, 1980). As a result, efflux of intracellular K+ is suppressed and calcium influx is enhanced, leading to an increase in neurotransmitter release and therefore, to an increase in nervous signalling (Molgo et al., 1985). In order to study the anticonvulsant role of chrysin on the excitatory neurotransmitter glutamate, NMDA (N-Methyl-D-aspartic acid)-induced lethality test was carried out, it is a most commonly used test to explore the possible role of glutaminergic pathway in the anticonvulsant effect of the test drug. In the present study, none of the tested doses of chrysin showed significant protection against NMDA-induced behavioral changes, However, the reference standard, promethazine (80 mg/kg) showed significant protection against NMDA-induced mortality in mice,

thus confirmed the negligible role of glutaminergic pathway in the anticonvulsant effect of chrysin. With the above finding it can be hypothesized that, the chrysin is eliciting potent anticonvulsant activity by increasing the inhibitory neurotransmitter (GABA). As a well-validated preclinical model, the maximal electroshock mimics the petit mal epilepsy observed in clinic. In principle, the electroshock delivered in MES model is well known to potentiate the sodium influx by opening of sodium channels, and also increases glutamate levels, glutamate is an excitatory neurotransmitter, and it binds to NMDA receptors and induces the symptoms that exactly mimic the petit mal epilepsy in humans (Carter et al. 2000). With the detailed understanding of underlying mechanism, it is stated and scientifically proved that, the agents which shows protection in this model will act either by blocking the voltage-dependent sodium channels (phenytoin, sodium valproate,) and/or by decreasing excitatory amino acid levels and/or by antagonizing their actions (e.g., felbamate) (Viswanatha et al. 2013). Chrysin did not show significant increase in anticonvulsant activity, in comparision to phenytoin (25 mg/kg), which abolished the MES-induced convulsions and mortality completely (Carter et al. 2000). In addition, it permits the evaluation of the ability of a substance to prevent seizure spread through neural tissue (Castel-Branco et al., 2009). Chrysin produced no anticonvulsant effect against MES-induced tonic seizures in mice.

Pentylenetetrazole kindling is a well-established animal model of generalized epilepsy. Chronic administration of PTZ in rodents at subconvulsant dose results in induction of kindling that mimics clinical epilepsy (Shimada and Yamagata 2018). It is the most extensively acknowledged animal model used to explore seizure mechanisms, understanding the neurobiology of epilepsy, learning and memory deficits induced by seizures and to discover the effectiveness of novel anti-epileptic compounds (Dhir 2012).

Experimental evidences clearly demonstrated that flavonoids exerts antiepileptic activity by modulating the $GABA_A$-Cl^- channel complex, due to their structural similarities with benzodiazepines (Avallone et al., 2000). Flavonoids isolated from plants are used as sedatives and tranquilizers, since they possess remarkable activity for ($GABA_A$) receptors and some of these possess pharmacological profile compatible with the above (Medina et al., 1998). Our earlier research established the richness of EPP in polyohenolics (Sharma et al., 2017). Polyphenolic analysis of EPP showed the presence of several polyphenols such as rutin, myricetin, chlorogenic acid, syringic acid, chrysin etc. Interestingly, chrysin was found to be one of the major polyphenol (26.06±0.21mg/gm) in the abovementioned extract. In our study, EPP (200mg/kg; *p.o.*) and chrysin (5mg/kg; *p.o.*) pre-treatment ameliorated seizure activity as indicated by low seizure scores in PTZ-kindled mice. Moreover, both extract and its major bioactive component showed almost equal efficacy in terms of reduction in seizure severity, indicating the fact that anticonvulsant activity of the EPP may be due to chrysin.

Numerous lines of findings has bolstered mitochondrial dysfunction for the pathogenesis of various neurological disorders (Picard et al., 2016). Since neurons have high-energy demands and no significant capacity to regenerate, this critical requirement makes them vulnerable to mitochondrial dysfunction. Mitochondria, in turn, are the primary site of reactive oxygen species (ROS) making them particularly susceptible to macromolecule dysfunction and oxidative damage (Turrens 2003). The latter could contribute to enhance neuronal excitability and increase seizure susceptibility (Heinemann et al., 2002). Mitochondrial dysfunction is a common trigger for apoptosis, as ROS damage can induce Cyt *c* release and sequential activation of pro-apoptotic caspase-9 and caspase-3 (Hong et al., 2016). Therefore, antioxidants that protect mitochondrial targets and decrease neuronal death may be useful supplements for the clinical management of

patients with seizures (Ullah et al., 2012). Our results revealed that pre-treatment with EPP 200mg/kg*; p.o*. and chrysin 5mg/kg; *p.o.* decreased PTZ-induced mitochondrial ROS production and suppressed neuronal apoptosis, possibly through the protection of mitochondrial function and the inhibition of Cyt *c* translocation and caspase activation. In order to further confirm the molecular change in the mitochondria that is responsible for the protective effects of EPP and chrysin pre-treatment, we focused on hitological observation. The findings from propidium iodide staining were consistent with the molecular results. Western blot analysis demonstrated that PTZ-induced seizures significantly increased the expression of active caspase-3 and caspase-9 in hippocampus. Propidium iodide staining indicated significant pyramidal cell loss with ultrastructural signs of apoptosis. EPP and chrysin pre-treatment reversed these morphological changes. The results of the present study corroborated with the earlier findings chrysin (2.5, 5, and 10 mg/kg; *p.o.*) significantly attenuated PTZ-induced changes in all the oxidative stress biomarkers in the selected rat brain regions. Therefore, chrysin could ameliorate the convulsion-induced oxidative stress in the mice brain. Which revealed antioxidant potential of chrysin. Considering all of the above, targeting mitochondrial bioenergetics and oxidative stress with EPP/chrysin may prove to be useful for the management of epilepsy

Earlier reports have revealed that chrysin alleviated the aged-related memory impairment in mice (Souza et al., 2015), but its neuroprotective effects with respect to seizures have not been previously investigated. Initiation of kindling leads to neuronal loss in the hippocampus that is associated with cognitive impairments, emotional changes and alteration in various other vital functions in experimental animal (Pahuja et al., 2013). In the present study, we found PTZ kindling resulted in learning and memory deficits in mice as evidenced by a significant increase in escape latency in training sessions. PTZ kindled mice exhibited much shorter time spent in the target

quadrant in the probe tests in MWM. EPP/chrysin treated PTZ kindled mice had improved performance in identification of submerged platform in the training sessions and also spent significantly longer time span in the target quadrant looking for the platform. However standard drug diazepam also showed significant increase in escape latency, indicating the fact that chronic use of diazepam as antiepileptic can lead to memory impairment. These observations suggest that EPP and chrysin improved cognition of PTZ kindled mice, contrary to standard drug diazepam. Thus EPP/chrysin mediated reductions in seizure scores and neuronal loss could have contributed to better cognitive abilities.

Neurotrophins play a very crucial role in development of the nervous system in the vertebrates. BDNF is classified as the second member of the neurotrophin family after nerve growth factor (NGF) which elicits its effects on both excitatory and inhibitory neurotransmitter functions by tropomyosin receptor kinase B-extracellular signal-regulated kinase (TrkB-ERK)-mediated phosphorylation of synapsin (Scharfman et al., 2005). The structures associated with epilepsy, primarily the cortex and hippocampus express TrkB receptors. It is believed that BDNF is associated with maintaining the synaptic transmission, neuronal plasticity and excitability (Mabuchi et al., 2001). Epileptogenic insults increase BDNF synthesis and TrkB receptor activation (Binder et al., 2001). Many studies convincingly support the notion that these phenomena have a proepileptogenic role (Liu et al., 2013). The previous in vitro and in vivo study has showed that, during PTZ induced acute seizure, BDNF-TrkB signaling pathway is activated to promote the epileptiform activities in hippocampal neurons (Wang et al., 2009). However, our current results demonstrated that BDNF decreased significantly in the hippocampus in chronic seizure phase in PTZ kindled animals. The western blot analysis showed increase in the hippocampal BDNF level in the EPP/chrysin group in comparison to PTZ group.

The protein expression results revealed that, PTZ administration reduced CREB and pCREB levels in the hippocampus of kindled animals. CREB has been identified as the key mediator for BDNF-mediated cell survival as previous studies showed that silencing the transcriptional activity of CREB impaired BDNF protection (Bonni et al., 1995). CREB is a key transcription factor that plays a role in several critical functions of the brain, such as learning, neuronal plasticity and cell survival (Sakamoto et al., 2011), pCREB promotes the transcription of immediate-early gene mRNA, which is then translated into proteins. These proteins are necessary for the maintenance of long-term memory (Segal and Murphy 1998). To identity the upstream regulators of CREB signaling, previous studies have demonstrated that different signaling can trigger intracellular signaling cascades that phosphorylate CREB at Ser133, which is a rate-limiting step in CREB signaling (Yamashima 2012). In the current study, we measured the BDNF, pCREB, CREB levels in the hippocampus region of mice brain after attainment of kindling i.e. day 15. Our results suggested that EPP-200 and chrysin-5 mg/kg upregulated the levels of BDNF, pCREB, CREB. The above evidence indicated that the elevated CREB activity subsequently markedly increased BDNF protein levels in the EPP/chrysin supplementation group, which was also associated with the recovery of spatial learning and memory.

Chrysin, a flavonoid compound isolated from EPP, is a positive allosteric modulator acting on GABAARs, in this study, we proposed that chrysin as a pharmacotherapy for epilepsy is two-fold, it affects plasticity of GABAergic transmission and through the mechanism of restoring gephyrin it keeps functional neurosynapses in the brain. Loss of functional GABAergic synapses can be part of the total loss of synaptic transmission leading to imbalance of neuronal excitation and inhibition, as well as abnormal communications between neurons. Gephyrin is the key inhibitory scaffolding protein. It organizes the postsynaptic receptor density at GABAergic and

glycinergic inhibitory synapses. Gephyrin anchors postsynaptic $GABA_A$Rs to regulate their formation and plasticity by clustering receptors in postsynaptic scaffolds and thereby regulating the availability and function of inhibitory receptors (Sheng and Kim 2011). In this study, we showed that gephyrin levels were upregulated in extract and compound treated mice compared to negative control mice. Therefore, gephyrin can be a novel target for treatments of epilepsy.