Chapter-6

Summary & Conclusions
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Sleep is very much essential for human or, animals for their normal, healthy function and for nervous systems to work properly. GABA (Gamma Aminobutyric acid) is the principal anti-neurotransmitter in the central nervous system of all mammalian. It plays a key part in the control of neuronal fieriness by preventing or reducing certain nerve signals (Smith & Simpson 2003). Decrease in GABA involved in pathogenesis of several neurological disorders. Drugs that enhance GABA activity are used for treating sleep disorders. Agents acting on GABA receptors have extensive therapeutic prospects including therapy of sleep, anxiety, depression and memory disorders (Chebib et al. 2006). One way of combating sleep disorder is by increasing GABA level. To fulfill the requirement, drug acting selectively on GABA receptor having novel sleep-promoting property is required. There are various classes of natural products including food constituents with hypnotic activity have been reported but have not been convincingly proved. Moreover, in Western countries, studies on the hypnotic effects of medicinal plants have been widely carried out, whereas in Asia, there are only few reports on the hypnotic effects of Asian plants.

The aim of this study is to find the efficacy of selected herbs for sleep disorder. To fulfill the aim an objective of the present studies includes:

- Authentication of Valeriana jatamansi, Valeriana wallichii DC and Withania somnifera Linn.

In vivo Studies

- Toxicity study of plant extracts
- Efficacy study
  - Sleep time measurement
  - Walking assay
- Estimation of phyto-constituents in plasma samples after dosing

In vitro Studies

- Estimation of GABA level by HPTLC
- GABA-A α1 subunit expression (Western Blotting)
A morphological examination which includes macroscopic and microscopic characterization is preliminary study to decide authenticity of drug. Physicochemical analysis, phyto-chemical analysis, fluorescence analysis gives qualitative information about the purity and the standard of the crude drug and presence of different functional groups in drug. The HPTLC fingerprinting profile is one of the very significant parameter for standardization of herbal drug and for appropriate authentication of the medicinal plants. Valproic acid an important constituent of Valeriana species and Withanolide A is steroid found in *Withania somnifera* were quantified using 10 ppm standards in all extracts. The results of the study provided correct identification of the crude drug and establish valuable source of information and suitable pharmacopeia standards for identification and authentication of this plant material for further applications.

Under safety study all extracts were found to be non-toxic up to the dose of 2000g/kg b.w (body weight). Irwin test showed decrease behavioral response and neurologic response in terms of muscle tone for extracts at 500 mg/kg b.w. The reduction of sensorimotor responses and motor- affective response indicates an action similar to drugs such as major sedative hypnotics(Navarro et al. 2011). Behavior observed was restored within 2-3 hour. Thus, indicating extracts may have short term effect.

Phenobarbitone induced sleep behavior is preliminary experiment to know dose dependent response of extracts to sleep behavior. Three doses for this study were taken (125, 250, 500 mg/kg b.w.). The result of induced sleep behavior study demonstrated that oral administration of extracts produced a dose-dependent action on sleep behavior i.e, decrease in sleep latency and an increase in sleep duration in animals pre induced with Phenobarbitone. The change in pentobarbital-induced sleep time is a useful experiment for examining effects (stimulatory or inhibitory) on the CNS particularly on the GABAergic system (Navarro et al. 2011) & (Liao et al. 1998). Phenobarbitone also known as phenobarbital is known to potentiate the effects of GABA, acting at GABA receptors (Navarro et al. 2011) & (Ticku & Maksay 1983). In this study aim was in find whether the prolongation of sleep behavior in animals’ received extracts may be due to an effect on the Central nervous system via the GABAergic systems along with Phenobarbitone. According to reference compounds/ drug which will increase the sleep duration and
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decrease the sleep latency over Phenobarbitone will also follow the same path and may be due to its effect on GABAergic system (Ma et al. 2008). There are herbs reported to be having sleep promoting activity by GABAergic system (Johnston et al. 2009) & (Khom et al. 2007). Any substance which induce sleep behavior activate GABA receptor either by directly acting on it or by positive modulation (Johnston 2005). Results were showing significant increase in sleep duration and decrease in sleep latency with all extracts effectively at 250 and 500 mg/ kg b.w. Since, the data indicates that Phenobarbitone-induced sleeping behavior was potentiated via the GABAergic systems selected extracts of Valeriana jatamansi, Valeriana wallichii DC, Withania somnifera Linn might influence GABAergic systems.

Walking assay (number of foot slips) is a very sensitive test to measure motor coordination and effective sedative dose (Stanley et al. 2005). Standard drug phenobarbitone significantly impaired motor coordination. Extract exhibited a non-significant reduction in the number of foot slips in non-dose dependent manner relative to Phenobarbitone treated group. The result of previous behavioural spontaneous motor activity and phenobarbitone induced sleep activity strongly suggests CNS activity of the extracts which also supports similar kind of other study (Dhingra & Goyal 2008). The extracts were also not showing any observable effects on motor coordination in the walking experiment which is signifying that the sleep effects observed are centrally.

GC-MS is widely accepted method for the analysis of phyto-constituents of herbal drugs preferabably volatile constituents. This method is ideal as it is highly sensitive, stable and efficient. Combination of GC with mass spectrometry (MS) has added the identification of compounds any complex mixture with more reliable information (Teo et al. 2008). In recent years much work has been done on GC hyphenated with MS for the analysis of phytoconstituents such as thymol, eucalyptol, menthol, estragole, safrole, eugenol, methyl, sugars etc (Patra et al. 2010).

Valerian is the common name given to the crude drug consisting of the underground parts of species of Valeriana. Valerians are known to be reservoir of mixture of essential oils. Complex mixture are mostly comprises of rich volatile components like lipids, terpenoids, ketones, phenols, oxygenated derivatives like antimicrobial, insecticidal and antioxidants (Burt 2004). Valeriana jatamansi was used as traditional medicine for the treatment of
anxiety disorders (H. Z. Zheng 2012) and now also anxiolytic property have been reported and used in clinical prescription (Yan et al. 2011). Valerian continues is preferred for mild to moderate sleep disorders (Hadley & Petry 2003). GC-MS screening of the essential oil of the *Valeriana wallichii* mostly consists of sesquiterpenes, terpenes like maaliol, santalene, acoradiene, β-gurjune and guaiol which together may be responsible for antinociceptive analgesic effect (Sah et al. 2010) & (S.P. Sah 2012). According to some reports *Valeriana jatamansi* contains iridoids, and flavonoids as components of essential oils (Lin et al. 2010) & (Bhatt et al. 2012), but the active compounds of *Valeriana jatamansi* which target directly for sleep disorder mainly insomnia have not been adequately elucidated. Some report states that valtrate at a high dose gives sedative properties by inhibiting spontaneous motion and increasing the sleeping number induced by pentobarbital sodium in mice (Chen et al. 2003). But the proper mechanism has not been reported.

In this study quantitative identification of volatile component in plant extracts was done by directly injecting plant extracts in GC column. In *Valeriana jatamansi* hexane extract total 44 and in methanolic extracts 38 chemical constituents were identified by matching with NIST.LIB library, literature about the previous reports on the selected plants. Similarly for *Valeriana wallichii* DC 38 compounds were matched and identified in hexane extracts and 43 compounds in methanolic extracts. According to our previous study on sleep time measurement it was observed that variability of concentrations of all extracts is correlated with the sleep. It was also observed that maximum concentration of extracts i.e. 500 mg/kg b.w. was showing its effect up to 3-5 hours. It was found that GABA levels were increased by using these extracts. According to literature studies crude plant extract of *Valeriana jatamansi* have been used in sedation, anticonvulsant, to treat hypertension and insomnia (YK. 1996) & (R. 2003) The plant consists of essential oils, iridoids and flavonoids (Li et al. 2013), but no specific compound is mentioned in literature which is responsible for any mentioned symptoms (Shi et al. 2014).

*Valeriana wallichii* DC contains various chemical constituents which are reported to act as an analgesic (Sah et al. 2010) & (S.P. Sah 2012). Based on literature the present study focused on to find out which components are responsible for sleep activity. Plant constituents were estimated in plasma samples collected at different time points using GC-MS. Maximum dose of 2000 mg/ kg b.w. was given to animals to get higher and better
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concentration in plasma and time points for blood collection were selected based on previous results in which effect of extracts in sleep activity was found. Components of plasma samples of each extracts were first normalized using blank plasma. Blank plasma was from animal which was administered with 0.5% CMC. Further blank normalized components of test sample (plasma sample with extracts) were matched with plant constituents (Results given). Compounds detected in extracts were matched with references (Liu et al. 2013). Plasma sample of animals administered with VJSHE, VJSME, VWSME were showing presence of Hexadecanoic acid, octadecanoic acid. Animal administered with VWSHE plasma sample showing presence of eicosane, tetracosane, octadecane, n-hexadecanoic acid, octadecanoic acid, henicosane. Compared to methanolic extracts hexane extracts administered test samples were showing more compounds. Compounds detected in plasma might be probable compounds responsible for sleep.

As the active constituents of Valeriana jatamansi and Valeriana wallichii DC are volatile GC-MS was selected for their analysis. In case of Withania somnifera Linn most of active constituents for treating neurotropic diseases are polar and GC-MS is not preferred for polar compound identification so, we developed HPTLC method for the identification. The study relates to a determination of Withaferin A from rat plasma by High Performance Thin Layer Chromatographic Method. Withaferin A was detected from plasma sample by using HPTLC method has been reported here first time. Withaferin-A is a bioactive constituents reported as promising drug candidates in neurological disorders (Patil et al. 2013). Withaferin A can be used as anxiolytic drug in place of diazepam that has lots of side effects (Khan & Ghosh 2010). For Withania somnifera Linn Linearity range was 50-600ng with $R^2=0.9621$. Withaferin A was present in plasma sample from 10 minute to 120 minute. Maximum concentration was 400.24ng/ml at 10 minute. Further there was decrease in concentration at 20 and 30 minutes. After 60 minutes saturation in concentration was found. The adopted high performance thin layer chromatographic (HPTLC) method is capable of evaluating withaferin A in plasma after extraction. The method could be used to carry out pharmacokinetic studies of the Withaferin A.

To determine GABA level HPTLC method was developed and validated. The method developed is specific for GABA because it resolved standard well in the presence of other amino acids in brain homogenate. The method was found to be suitable for both qualitative and quantitative analysis of GABA in the brain homogenate of mice. The
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mentioned HPTLC method is sensitive as analyte can be detected at nanogram concentrations with good visible bands, LOD being 50 ng and LOQ, 151 ng. Thus, the method was found to be simple, specific, accurate, sensitive, and precise. Furthermore, the method can be used as diagnostic tool for many neurological diseases.

To determine the potential of extracts of *Valeriana jatamansi*, *Valeriana wallichii* DC and *Withania somnifera* Linn for sleep disorder treatment was investigated through positive allosteric modulation on GABA receptor. HPTLC method proposed was used to determine GABA. According to results all extracts of *Valeriana jatamansi*, *Valeriana wallichii* DC, and *Withania somnifera* Linn exhibited enhanced effects on GABA receptor.

In vivo testing also revealed a noteworthy capacity of extracts of *Valeriana jatamansi*, *Valeriana wallichii* DC, and *Withania somnifera* Linn for inducing sleep. Sedative-hypnotic effects based on positive allosteric modulation of GABA-A receptors has been widely reported for the medicinal plants such as valerian (Shrestha et al. 2012). Indeed, GABAergic neurotransmission plays a key role in sleep regulation (Johnston 2005). The effect of VJSME, VJSHE, VWSMW, VWSHW and WSSME on level of GABA Receptor with respect to control was demonstrated. Animals treated with VJSME and VJSHE are showing increase in level of GABA with respect to control. Similarly for VWSME and VWSHE level of GABA was found to be higher than control. Even though the increasing trend in level of GABA was observed but the level of GABA was significantly high at 500 mg/kg b.w dose level for all extracts. In case of WSSME significant increase in level of GABA was observed in both dose level but at higher dose (500 mg/kg b.w.) GABA level was slightly decreased because of binding inhibition. *Withania somnifera* extract contains an ingredient which has a GABA-mimetic activity (Mehta et al. 1991). Methanolic extracts of *Withania somnifera* produces dose dependent inhibition of GABA and increase the specific binding of $^{(3)}$H flunitrazepam. This causes lowering total GABA but as fluronitrazepam is also giving sedative effects so behavior effects remain same.

Therefore, it can be suggested that VJSME, VJSHE, VWSME, VWSHE and WSSME may play a key role in the sleep inducing effect by activation of GABA. Phenobarbitone, positive control acting directly on GABA-A receptor, also increased the level of GABA. Extracts have also potentiated the onset of sleep along with extended the sleep time in animals suggesting that extracts have produced central effects in rodents, Moreover, since
pentobarbitone-induced sleeping behaviour was potentiated via the GABAergic systems, and also there was increase in GABA level after treatment with all extracts with respect to control data indicates that extracts might be acting on GABA.

Glutamic acid/ L- Glutamate is the main component for the synthesis of the inhibitory GABA. Transamination of α-ketoglutarate changed into L-glutamic acid and GAD further catalyzes the decarboxylation of glutamic acid to form GABA(Olsen & Guo-Dong Li 2011). There should be always balance between GABA and L- Glutamic acid to maintain the physiological state. GABA is metabolized by GABA-T and forms succinic semi-aldehyde (SSADH), this transamination, reforming glutamic acid. The whole process is closed loop process (Olsen & DeLorey 1999). Increase level of GABA may be because of directly synthesis of GABA from L- Glutamic acid. So, the change in level of Glutamic acid was checked using HPTLC method under same chromatographic conditions which were used for GABA. As per our result VJSME, VJSHE and WSSME the level of GABA and glutamic acid are positively correlated. So it can be said that VJSME, VJSHE and WSSME might have some effects on synthesis of GABA. In case of VWSME and VWSHE there is negative correlation between GABA and glutamic acid at higher dose (500 mg/ kg b.w). As there is always internal balance between GABA and L- Glutamic acid level (Wiero et al. 2011) and there is at least five mechanism by which GABA level increases(VCU 2002)(Ketter et al. 1999), including reuptake mechanism (Santos et al. 1994) and inhibition of catabolism of GABA (Riedel et al. 1982). In case of VWSME and VWSHE high level of GABA might be contributed by other mechanism along with synthesis mechanism. Other studies have shown that the root extract of valerian plant has valeric acid, which is a GABA degrading enzyme inhibitor (Houghton 1998). VWSME and VWSHE extract also contains valeric acid in their extracts as reported through GC-MS data. So it can be concluded that simultaneous increase in GABA and L-Glutamic acid level indicate extracts might have some effects on synthesis of GABA.

The Western Blotting study provided the first direct evidence that administration of extracts of Valeriana jatamansi, Valeriana wallichii and Withania somnifera increases the level of a GABA-A receptor subunit, the α1 subunit in brain. Earlier report shows that sedative-hypnotic effects and sleep activities are mediated by GABA-A receptors that contain the α1 subunit (Mohler 2006). A benzodiazepine and non-benzodiazepine drug which induces behavioral sedation in mice is evidently attributable to enhanced level of
GABA neurotransmission which is mediated by generally α1 subunit (Rudolph et al. 1999), (McKernan et al. 2000), (Kralic et al. 2002) & (Blednov et al. 2003). Drugs which are being used for treatment of insomnia like diazepam (Rudolph et al. 1999) & (Löw et al. 2000), Indiplon which is nonbenzodiazipine positive allosteric modulators (Petroski et al. 2006) and used as sleeping pills have been been reported to mediate the sedative effects via GABAA receptors mostly α1 subunits.

The expression of α1 subunit was tested after administration of 250 and 500 mg/kg b.w extracts of plant extracts of VJSME, VJSHE, VWSME, VWSHE, WSSME and Phenobarbitone 60 mg/kg b.w. We found all extracts potentiated α1 subunit, containing GABAA receptors at 500mg/kg b.w. dose level but had no effect on control group and VWME (data shown). In this study whole brain homogenate was taken due to which detectable level of GABAA α1 subunit was not found in control. Some studies have shown expression of GABA-A α1 subunit but they have used specific region of brain and GABA enrichment method (Benders 1989) & (Pal 2009). Phenobarbitone a standard drug used for insomnia and targeted for GABA receptor also has shown enhanced expression of GABA-A α1 subunits.

DAB was used in first attempt for development but it failed as DAB has very low sensitivity. Further TMB was used for the same and result came with very faint bands. To get clear picture about expression finally ECL was used and which gave a very prominent band (Results given). Although the present data may not prove a pivotal role for α1 variations in the insomnia or sleep disorder, but they suggest that increased α1 levels after administration of extracts may help in treatment of sleep disorder.

It must be noted that the presented study is short-term study and cannot based on it it can not be determined that enhanced α1 subunit expression is solitary responsible for treating sleep disorder; some other receptor in synergism or mechanism is contributing so final determination will require a much longer-term study. It can be concluded from results that expression of GABA-A α1 subunit suggests extracts might be acting selectively on GABA-A α1 subunit which is responsible for sleep.