The role of herbal medicine in the treatment of various physiological disorders has become well established over the past two decades. The beneficial medicinal or pharmacological activity of plant materials typically result from individually or in combinations of secondary metabolites present in the plant, through additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process (Briskin, 2000). This fact has a basis in the sense that medicinal actions of plants are unique to particular plant species or groups, consistent with the concept that combinations of secondary metabolites in a particular plant are often taxonomically distinct (Wink, 1999). It has been established that there are lot of plant secondary metabolites being employed in the treatment of psychotic disorders especially for anxiety in traditional medicine practice, most of which directly or indirectly affect the central nervous system (Wolfman et al, 1994; Salgueiro et al, 1997; Paladini et al, 1999; Dhawan et al, 2003).

Despite the widespread traditional use of selected plants (Dalbergia sissoo Roxb., Citrus limon Linn. and Elaeocarpus sphaericus) for treating various disorder including CNS. There is no scientific work has been conducted on the anxiolytic activity of bark of selected plants, therefore the present study was aimed to explore pharmacognostic and pharmacological potential of these selected plants.

Standardization is a very important tool in the quality control process of medicinal plants and it distinguish the authentic plant sample from its adulterant substitute. The present study showed macroscopy, microscopy, ash value, extractive value, determination of heavy metals and microbial count for Dalbergia sissoo Roxb., Citrus limon Linn. and Elaeocarpus sphaericus stem bark.

The organoleptic evaluation confirmed the colour, taste shape, size, and odour etc. of the bark
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Powder microscopy showed the diagnostic characters.

The ash value of the selected plant materials help to detect the adulteration with inorganic impurities and results showed that selected plant materials were devoid of any contamination, substitution or adulteration.

The extractive value obtained by exhausted plant material with ethanol and water was an indicative of approximate measure of their chemical constituents extracted out with these solvents from a specified amount of air dried plant.

Contamination of medicinal plant material with heavy metals can be attributed to many causes including environmental pollution and traces of pesticides (WHO, 2004). Selected plant materials were found to possess traces of heavy metals that were within the prescribed limits.

Plant materials normally carry a great number of bacteria and moulds often originating in soil. While a large range of bacteria and fungi from the naturally occurring microflora of herbs (WHO, 2004). The estimation of microbes is a valuable parameter to determine the quality of drug. Selected plant extracts were found to be free from microorganism.

However, the anxiolytic effect was evidenced by the EPM test that has been recognized as a valuable model able to predict anxiolytic effects of drugs in rodents (Perez, 1998). The EPM animal model is considered one of the most widely validated tests for assaying anxiolytic substances such as the benzodiazepines (Pellow and File, 1986). The fear due to height (ACROPHOBIA) induces anxiety in mice when placed on the elevated plus maze. The ultimate manifestation of anxiety and fear then is exhibited by decrease in motor activity, which is measured by the time spent by mice in the open arms. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration (Bourin and Hascoet, 2003).
Amongst various selected plant extracts, only methanol bark extract of *Dalbergia sissoo* Roxb. & *Citrus limon* Linn. and chloroform bark extract of *Elaeocarpus sphaericus* significantly increased mean number of entries and mean time spent by mice in open arms of elevated plus maze apparatus at different doses with respect to control, thereby producing anti-anxiety activity. When the animals were exposed to the EPM test, all mice were sensitive to methanol bark extract of *Dalbergia sissoo* Roxb. (at all dose *i.e.* 100, 200, 400, 800 mg/kg), methanol bark extract of *Citrus limon* Linn. (at dose 200, 400, 800 mg/kg) and chloroform bark extract *Elaeocarpus sphaericus* (at dose 800mg/kg only). At highest dose (800 mg/kg) of methanol bark extract of *Dalbergia sissoo* Roxb. and *Citrus limon* Linn. and chloroform bark extract *Elaeocarpus sphaericus* exhibit maximum significant (*p*<0.05) anxiolytic effect that was also produced by diazepam (at dose 2 mg/kg) but out of three selected plants the maximum anxiolytic activity is obtained by methanol bark extract of *Dalbergia sissoo* Roxb. at dose 800mg/kg showed a similar behavior to that of mice treated with DZP at dose 2 mg/kg (there were no statistical differences between these two groups). The methanol bark extract of *Dalbergia sissoo* Roxb. showed significant increase in the number of entries and time spent in the open arms of the elevated plus maze in a dose dependent manner. These results could indicate that amongst all selected plants extracts the maximum anxiolytic activity is shown by methanol bark extract of *Dalbergia sissoo* Roxb.

The anxiolytic effect was also evidenced through two compartmental exploratory model (light-dark test) was originally described by Crawley and Goodwin (1980) and been validated pharmacologically, behaviorally and physiologically (Crawley, 1981; Chopin and Briley, 1987). This test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, *i.e.* novel environment
and light. As with the EPM test, this model is useful for modeling anxiety, and it has been developed for predicting the potency of clinically used compounds for treating this disease. It has been assumed that the time spend by mice in the illuminated side of the box is the most useful and consistent parameter of anxiety (Young and Johnson, 1991). As expected, diazepam produced significant increase in the time spent and no. of entries in illuminated side box of light and dark apparatus test. The result of light and dark test model showed the time spent by mice in light compartment was significantly increase by methanol bark extract of *Dalbergia sissoo* Roxb., *Citrus limon* Linn. and chloroform bark extract of *Elaeocarpus sphaericus* as same as with EPM but maximum significant (p<0.05) result received at dose 800 mg/kg of all the above said extracts. Amongst three significant extracts, the maximum significant result (anxiolytic activity) produce by methanol bark extract of *Dalbergia sissoo* Roxb. at dose 800 mg/kg that is nearly equal to diazepam (at dose 2 mg/kg) results.

Ideally actophotometer used for monitoring accessing exploration and locomotor activity in rodents. Locomotor activity is considered as an index of alertness and a decrease in that indicates a sedative effect. Following administration of methanol bark extract of *Citrus limon* Linn. and chloroform bark extract of *Elaeocarpus sphaericus* (800 mg/kg) in mice showed significant decrease in the locomotor activity when compared to control that is similar with diazepam effect. Diazepam and related compounds bind to the GABA<sub>A</sub> receptors in CNS leading to sedative effects. In the locomotor activity study, the decrease in locomotion caused by CNS depression may be due to the increased concentration of GABA in the brain (Pellow, 1985). These results show that both extract and diazepam have sedative effect by increased concentration of GABA in the brain. But methanol bark extract of *Dalbergia sissoo* Roxb. at dose 800 mg/kg showed significant increase in the locomotor activity when compared to control. This result indicate
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alertness or stimulation of CNS.

Preliminary phytochemical screening is one of the initial and necessary step to find out the nature of phytoconstituents present in the extract of the plant, which further leads to the isolation of active compounds. In this study, preliminary phytochemical analysis revealed the presence of flavonoids, carbohydrates, tannins and phenolic compounds in the methanol bark extract of *Dalbergia sissoo* Roxb. Flavonoids and phenolic compound in the methanol bark extract of *Citrus limon* Linn. and alkaloids and flavonoids in the chloroform bark extract of *Elaeocarpus sphaericus*.

Medicinal plants used in the folk medicine may be an interesting and largely unexplored source for the development of potential new compounds (Lindequist *et al.*, 2005), but it is necessary to isolate the active principles and characterized their constituents for the beneficial of human being. In a preliminary anxiolytic screening the methanol bark extract of *Dalbergia sissoo* Roxb. was chosen for isolation of compounds because it contained more potent anxiolytic compounds (anxiolytic activity) than other extracts.

Column chromatography is one of the most frequently used techniques in the isolation of natural plant constituents. The Column chromatographic separation of methanol bark extract of *Dalbergia sissoo* Roxb. (with most significant anxiolytic effect) led to the isolation of two bioactive compounds *i.e.* Compound A and B were sufficiently pure and shows single spot on TLC with $R_f$ value 0.55 and 0.72 respectively.

**Anxiolytic activity of Isolated Compounds** – In EPM, naive mice will normally prefer to spend much of their allotted time in the closed arms. In this study, we observed that compound-A (at dose 2, 5, 10 mg/kg) and compound-B (at dose 10 mg/kg only) induced significant increases in the both number of entries and time spent in the open arms and the number of entries and time
spent in the closed arms were reduced in the EPM model. The maximum anxiolytic activity of compound-A showed at dose 10mg/kg when compared to control.

The above result was also supported by another anxiolytic model *i.e.* Light and Dark test model. The Light Dark test has been widely adopted as an anxiolytic screening test in mice (Costall, 1989). This method titrates a natural tendency of mice to explore a novel environment, against the aversive properties of a brightly lit compartment. In this study, compound-A (at dose 5 & 10 mg/kg) and compound-B (at dose 10mg/kg) significantly (p<0.05) increased mean number of entries and mean time spent by mice in illuminated side box of light with respect to control but maximum significant (p<0.05) effect of compound-A was received at dose 10 mg/kg thereby producing anti-anxiety activity.

Locomotor activity is a measure of the level of excitability of the CNS (Mansur *et al.*, 1980). This decrease in spontaneous motor activity could be attributed to the sedative effect of the plant extracts (Rakotonirina *et al.*, 2001). In present study, locomotor activity on actophotometer did not decrease by compound-A and Compound–B at all doses but significantly decrease by diazepam when compared with control group. Locomotor activity on actophotometer slightly increased by both compound-A and compound-B but significantly increased only by compound-A at dose 10 mg/kg when compared with control group. Thus compound-A does not producing sedative effect but shows anxiolytic activity with index of alertness.

Analysis of the neurotransmitter data supports the results of behavioral models. There is now considerable evidence to implicate the serotonergic system with anxiety. The serotonergic system has been implicated in the alterations in appetite, energy, sleep, mood, libido, and cognitive functioning seen in anxiety and affective disorders. Decreased serotonin levels in animal models and human studies have been associated with increased impulsivity, aggression,
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fear, and anxiety/depression. Furthermore, the improvement of symptoms following treatment with serotonergic antidepressants (e.g., SSRIs) has been one explanation supporting the role of serotonergic dysfunction in anxiety. Compound-A significantly (p<0.05) increased serotonin level in brain regions like frontal cortex, hypothalamus, hippocampus, and amygdala at dose 5mg/kg and 10mg/kg.

Low activity of serotonin may permit the dysregulation of other neurotransmitters, including norepinephrine (Ninan, 1999). It has been earlier reported that, naturalistic stimuli such as exposure to novel cage, cause sustain and significant increase in nor-epinephrine release in frontal cortex (Bhattamisra et al., 2007). Variety of stressful events, including emotional stress causes a marked increase in noradrenaline release in several brain regions, and especially in the hypothalamus, amygdala and locus coeruleus, in the mice brain. This suggests that an increased noradrenaline release could be closely related to the provocation of negative emotions such as anxiety and/or fear (Tanaka et al., 2000). Compound-A (10 mg/kg) which attenuate significantly (p<0.05) decrease of noradrenaline level in frontal cortex, hypothalamus and amygdala region of the brain that could have anxiolytic properties.

Dopamine is a catecholamine neurotransmitter which is a chemical in the body that works to send messages to the nerves cells, allowing them to communicate with each other. The high levels of dopamine can cause anxiety, paranoia, panic attacks or hyperactivity. High dopamine in anxiety could suggest that it is causing people to be motivated to ruminate about their fears. In this study compound-A (at dose 5 & 10 mg/kg) significantly (p<0.05) decrease dopamine level in brain regions like frontal cortex, hypothalamus, hippocampus, and amygdala when compared with control.
GABA appears to play an important role in the pathogenesis of several neuropsychiatric disorders. Many of the traditional agents used to treat psychiatric disorders are known to act, at least in part, by enhancing GABA activity, while some of the newer agents may exert their therapeutic effects exclusively via GABAergic actions (Pitchaiah et al., 2010). In present study, it was found that GABA was significantly (p<0.05) increased in cerebellum and whole brain other than cerebellum by diazepam (2mg/kg) when compared with control group. Diazepam and related compounds bind to the GABA_A receptors in CNS and increased concentration of GABA in the brain via GABAergic actions (Pellow, 1985). On the other hand, compound-A has not produced significant (p<0.05) increase in GABA levels in cerebellum and whole brain other than cerebellum of the mice when compared with control/normal group.

**Compound-A**: On the bases of extensive spectroscopic data analysis and by comparison their spectral data with those reported in literature compound-A determined as C_{16}H_{12}O_4 (m/z = 268.01). The UV spectrum exhibited absorption maxima at 277 nm that is characteristic flavonoids skeleton bands. The IR spectrum indicated the presence of hydroxyl group stretching (3367.71cm^{-1}), carbonyl function (1633.31cm^{-1}) and C-O-C groups (1170.79cm^{-1}). The presence of these common flavonoid aglycons was confirmed from ^1H NMR spectra. In ^1H NMR presence of hydroxyl group C-6 appeared as a sharp singlet at δ 5.758 and methoxy group at C-7 showed sharp singlet at δ 3.78.

The Compound A was characterized as 6-Hydroxy-7-methoxy-4-phenyl-chromen-2-one.

The characterized needle shape whitish yellow crystal melting point and UV, NMR and IR spectrum of compound-A suggested that it was a neoflavonoid derivative compound.

**Compound-B**: Compound was assigned the molecular formula C_{16}H_{14}O_3 (m/z = 254.09). The UV spectrum exhibited absorption maxima at 262 nm which is characteristic flavonoid skeleton
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band. The IR absorption at 1683.86 cm\textsuperscript{-1} suggested the presence of carbonyl group. The \textsuperscript{1}H NMR spectra showed the presence of sharp singlet due to the methoxy protons at $\delta$ 3.53 and the singlets appeared at $\delta$ 6.95 and $\delta$ 5.79 corresponding to aromatic protons present at 3 and 6 position of benzoquinone nucleus.

The Compound-B was characterized as 2-Methoxy-5-(1-phenyl-allyl)[1,4]-benzoquinone.

The characterized yellowish brown amorphous powder, melting point and UV, IR and NMR spectrum of compound-B suggested that it was the neoflavonoid derivative compound.

Flavonoids are the most important secondary metabolites in many plants that are held responsible for their anxiolytic actions without sedation (Paladini et al., 1999; Marder and Paladini, 2002). However, flavonoids (Compound-A and compound-B) as the major constituent of the methanol stem bark extract of Dalbergia sissoo Roxb. were observed anxiolytic action without sedation. Alteration effect on brain serotonin and noradrenaline/dopamine level induced sleep or sedation because high level of serotonin produces alertness or reduces sleeping time. On the bases of monoaminergic neurotransmitters results, the possible anxiolytic mechanism without sedation of methanol bark extract of Dalbergia sissoo Roxb. suggests that the compound-A act on serotonergic and dopaminergic system without effect on GABAergic system.

Conclusively, Compund A alone or in combination with Compound B (present in methanol bark extract of Dalbergia sissoo Roxb.) possess anxiolytic activity without sedation.