`

ASSESSMENT OF ANTIOXIDANT AND HYPNOTIC ACTIVITY OF UNANI FORMULATION ARQ GULAB

Upendarrao.Golla*, Ashok Kumar.K, Solomon Suder Raj.B

Department of Pharmacology, Bharat Institute of Technology (Pharmacy), Hyderabad, R.R.Dist-501510, Andhra Pradesh, India.

*golla.upendarrao@gmail.com

Summary

Complementary system of medicine includes namely Ayurveda, Siddha, Unani, Kaempo, and Chinese medicine has gained its popularity in recent years. Now Unani system of medicine became an integral part of Alternative medicinal systems of India. Arq Gulab is a clear aqueous distillate of flowers of the plant *Rosa damascene,* a Unani product recommended by the traditional practitioners in a variety of cardiovascular, nephro, neurodegenerative and other disorders. In the present study, Arq Gulab was evaluated for its antioxidant activity by using various *in vitro* models like 1, 1-Diphenyl, 2-Picryl- hydrazyl (DPPH) free radical, Nitric Oxide scavenging activity and reducing power methods and hypnosis activity by using Diazepam induced sleep model in mice. The results for hypnosis activity showed that the Arq Gulab significantly ($p< 0.01$) reduced the onset and prolonged the duration of sleep at the doses tested (3 & 6ml/kg) comparable to control and under *invitro* DPPH free radical and nitric oxide free radicals are considerably inhibited in a dose dependent manner and there is increase in absorbing power indicates increase in reducing power. This study revealed that the Unani formulation Arq Gulab possesses significant hypnosis activity and antioxidant activity and also our study justifies its therapeutic application and lends pharmacological credence to the ethnomedical use of Arq Gulab in Unani system of medicine.

Key words: Unani formulation, arq gulab, free radical, antioxidant, diazepam, hypnosis.

Introduction

Complementary system of medicine includes namely Ayurveda, Siddha, Unani, Kaempo, and Chinese medicine has gained its popularity in recent years (1). Unani system of medicine (unanipathy) originated in Greece based on the principles propounded by Galen. After him many Arab and Persian scholars enriched the system and became Unani (2). Now it has become a part of Indian traditional system of medicine (3). The demand of herbal medicine is increasing day by day due to their efficacy, rare chances of side effects in the treatment and good faith of society on herbal medicine and also their products (4).

Arqs are liquid preparations, obtained by the distillation of macerated crude drugs in aqueous medium (5). Different types of Arqs like Arq Gulab, Arq mundi, Arq kasni, Arq gauzaban etc., have been used by the traditional practitioners for the management as well as treatment of disorders related to various organs. Number of drugs, both single and compound preparations is used widely in Tibb-e-Unani (Unani medicine) in the management of various diseases. But such drugs mostly, have not been investigated for their described effects. Arq Gulab is one such formulation, is a clear, non-viscous liquid preparation obtained by the aqueous distillation of duly macerated flowers of the plant *Rosa damascena* Mill. belongs to the family *Rosaceae*. In Unani system of medicine, Arq Gulab was described to be refrigerant, reduces thirst, gives relief in conjunctivitis, anxiety, syncope, palpitation and provides strength and cheerfulness to heart. It is mainly prescribed when there is a weakness of the principal organs of the body like Brain, Heart and Liver.

Antioxidants are free radical scavengers which protect the human body against free radicals (6). Recently extracts of plants, Unani formulations have provoked interest as sources of natural products. They have been screened for their potential uses as alternatives medicines for the treatment of many infectious diseases and also in preservation of food from the toxic effects of oxidants. In modern days the antioxidants have formed the basis of many applications in pharmaceuticals, alternative medicines and natural therapy. Because of the possible toxic effects of synthetic antioxidants like-Butylated hydroxyl anisole (BHA) and Butylated hydroxyl toluene (BHT), an increased attention has been directed towards natural antioxidants (7).

Phytochemical literature reveals the presence of glycosides, reducing sugars, tannins, terpenes and volatile oils in this plant (8-10). This formulation is used internally and externally

to treat ophthalmic disorders, abdominal and chest pain and strengthening the heart, also for its hypnotic, antispasmodic effects by the local traditional practitioner for long time. Antispasmodic (11), anti-bacterial (12), ophthalmic care (13) and anti-HIV (14) effects of *Rosa damascena* flowers have been reported. Therefore in the present study, an attempt has been made to evaluate the Unani formulation of the *Rosa damascene* flowers (Arq Gulab) for hypnosis activity and invitro antioxidant activity which are of relevance to its application in folklore medicine. It's Hypnosis activity was investigated by using Diazepam induced sleep model in mice as there is no scientific evidence for its CNS activity and antioxidant activity under *in vitro* models like 1, 1-Diphenyl, 2-Picryl-hydrazyl (DPPH) free radical, Nitric Oxide scavenging activity and reducing power method as there is growing evidence of free radicals role in disease progression in number of diseases and concomitant antioxidant administration.

Materials and Methods

Animals

Swiss Albino mice (20-28 g) of either sex maintained at the institutional animal house were used. They were kept in standard polypropylene animal cages with 12 hr of light and dark cycle in a room with controlled temperature (22° ± 3°C) of the institutional animal house. The animals were fed with standard rodent's chow diet and provided water *ad libitum*. After one week of acclimatization the animals were used for further experiments. Approval for the usage of animals in the experiments was obtained as per the Indian CPCSEA guidelines outlined by the Institutional Animal Ethical Committee of the faculty and Approval number of Committee was 1015/c/06/CPCSEA.

Drugs

Arq Gulab (M/s. Hamdard Laboratories, Ghaziabad, India) was purchased from local retail Unani pharmacy and Diazepam (Calmpose^{R} Inj. Ranbaxy, India) was sourced locally.

Chemicals

L-Ascorbic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH) were purchased from Sigma Aldrich, USA. Potassium ferricyanide, Trichloro acetic acid, Ferric chloride, Sodium Nitroprusside, Glacial acetic acid, 1-Napthyl amine and all other chemicals and reagents used to carry out this research were obtained commercially from the regular store suppliers and were of analytical grade.

In Vitro Antioxidant Activity

DPPH Radical Scavenging Activity (15, 16, 17): DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diagnostic molecule. The reduction capacity of DPPH radical was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. 1ml of 0.2 mM DPPH solution in ethanol was added to undiluted Arq Gulab at different concentrations (0.5 & 1ml) and the mixture was vigorously shaken and allowed to stand at room temperature for thirty minutes. The absorbance was measured at 517 nm in a spectrophotometer. DPPH in ethanol was taken as control and procedure was repeated. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Experiment was done in triplicate. The difference in the absorbance between the test and the control was calculated and expressed as percent inhibition of DPPH radical. Percent radical scavenging activity was calculated by using the following formula.

% Radical Scavenging activity = [1 – Abs. of sample / Abs. of control] x 100

Nitric oxide radical inhibition activity (18): The nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH is inhibited by antioxidants which compete with oxygen to react with nitric oxide. Nitric oxide was measured by the Griess reagent. The reaction mixture (3 ml), containing sodium nitroprusside (10 mM, 2 ml) in phosphate buffer saline and different concentrations of undiluted Arq Gulab (0.5ml, 1ml) was incubated at 25°C for 150 min. After incubation, 1 ml of the reaction mixture containing nitrite was pipette out and 1 ml of Griess reagent (1% sulphanilamide, 2% O– Phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride) was added and allowed to stand for 5 mins for completion of diazotization. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with napthyl ethylene diamine (NED) was read at 546 nm against the corresponding blank solutions. Experiment was done in triplicate. Capability to scavenge the nitric oxide radical was calculated as percent inhibition by using formula.

% inhibition = [1 – Abs. of sample / Abs. of control] x 100

Reducing power Method (19, 20): Substances which have reduction potential react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+) which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. Various doses of the Arq Gulab (0.5ml &1ml) in 1.0 ml of deionized water were mixed with phosphate buffer

(2.5 ml, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). Absorbance was measured at 700 nm. A blank was prepared without adding Arq Gulab. Increased absorbance of the reaction mixture indicates increase in reducing power. % increase in Reducing Power was calculated by using formula.

% increase in Reducing Power = [(Abs. of test/ Abs. of blank) – 1] x 100

Hypnotic Activity

Diazepam-induced sleep in mice: The method described by Beretz *et al.* (21) and modified by Rakotonirina *et al.* (22) was adopted in this study. Hypnotic effect method based on potentiation of diazepam induced sleeping time by Arq Gulab. Adult mice of either sex weighing 20-28g were divided into three groups of six mice in each. The first group was administered normal saline (5ml/ kg), second and third groups were administered Arq Gulab at the doses 3ml/kg and 6ml/kg intraperitoneally. Thirty minutes later, Diazepam (3mg/kg) was administered via intraperitoneal route to all the mice to induce the sleep. The criterion for sleep is the loss of righting reflex, in which the mice cannot roll back when turned over (23). Each mouse was then observed for the onset and duration of sleep. The time interval between injection of Diazepam and start of sleep was recorded as onset (latency time) and the time interval between loss and recovery of righting reflex was recorded as duration of sleep. The interval between loss and recovery of righting reflex was used as index of hypnotic effect (24, 25).

Statistical analysis

The various values were expressed as Mean ± SEM. The data were statistically analyzed using ANOVA with multiple comparisons versus control group. The values of $p<0.05$ were considered as significant (26). The treated groups and control groups were analyzed separately for statistical significance.

Results and Discussion

In Vitro Antioxidant Activity

DPPH free radical scavenging activity: DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with

DPPH both transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and the degree of discoloration indicates the scavenging activity of the drug. The reduction capacity of DPPH radical is determine by the decrease in its absorbance at 516 nm induced by antioxidants by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow.

Arq Gulab at various doses produced inhibition of DPPH in dose dependent manner. The obtained results of absorbance and % inhibition showed decrease in the concentration of DPPH radical due to scavenging ability of extracts and at 0.5, 1ml of Arq Gulab exhibits 64.14, 89.47% inhibition which is considered significant when compared to control. The results were shown in **Table 1** and depicted in **figure 1.** The significant activity of Arq Gulab suggests that hydrogen donation maybe a possible mechanism for antioxidant activity of this formulation.

Table 1: DPPH radical scavenging activity of Arq Gulab Unani Formulation.

Volume of undiluted arq gulab used (ml)	DPPH radical scavenging activity	
	Absorbance at 517 nm	% Inhibition
0 (Control)	0.884	0
0.5	0.317 ± 0.002*	64.14
1	0.093 ± 0.002*	89.47

All values are expressed as mean ± SD (n=3). All values are significant at *P < 0.001 when compared to control.

Nitric oxide radical scavenging activity: Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrate ions that can be estimated by use of Griess reagent. Sulfanilamide is quantitatively converted to a diazonium salt by reacting with nitrite in acidic conditions. This diazonium salt coupled with 1-napthylamine; forming an azo dye that can be measured quantitatively at 540nm. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide.

The Arq Gulab showed good nitric oxide-scavenging activity, at 0.5, 1ml dose exhibits 37.16, 78.25% inhibition which is considered extremely significant when compared with control. The percentage of inhibition increased with increasing dose of the formulation. The results were shown in **Table 2** and depicted in **figure 1.**

Table 2: Nitric Oxide radical scavenging activity of Arq Gulab Unani Formulation.

Volume of undiluted arq gulab used (ml)	NO radical scavenging activity	
	Absorbance at 546 nm	% Inhibition
0 (Control)	0.869	0
0.5	0.546 ± 0.005*	37.16
1	0.189 ± 0.003*	78.25

All values are expressed as mean ± SD (n=3). All values are significant at *P < 0.001 when compared to control.

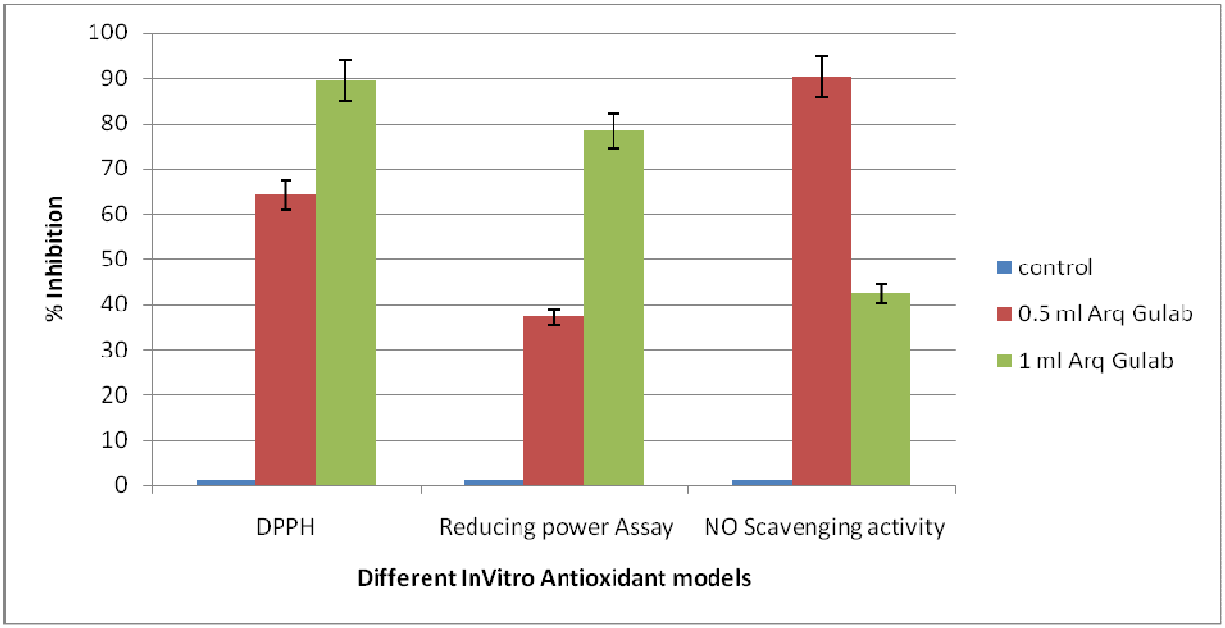
Reducing power assay: Substances which have reduction potential react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+). Potassium ferrocyanide then reacts with ferric chloride to form ferric ferrous complex. This complex has an absorption maximum at 700 nm. It showed 90.25, 42.4% Inhibition at o.5, 1ml respectively. As there is increase in absorbance indicated significant increase in reducing power when compared to control. It was found that the reducing power of Arq Gulab increased with the increase of dose. The results were shown in **Table 3** and depicted in **Figure 1.**

Table 3: Reducing power assay of Arq Gulab Unani Formulation.

Volume of undiluted arq gulab used (ml)	Reducing power assay	
	Absorbance at 700 nm	% Inhibition
0 (Control)	0.852	0
0.5	0.231 ± 0.002*	90.25
1	0.638 ± 0.002*	42.4

All values are expressed as mean ± SD (n=3). All values are significant at *P < 0.001 when compared to control.

Figure 1: % Inhibition of free radical by Arq Gulab formulation in DPPH, Reducing power assay and Nitric oxide scavenging methods.



It is quite apparent from the results compiled in the table-1 that, the formulation has satisfactory free radical scavenging activity. It is well known fact that, it is very difficult to determine the exact chemical composition and strength of the arqs; which constrained us to determine the antioxidant activity contained in minimum volume (1ml) of undiluted preparation, which can be further extrapolated to any volume that is used for the therapeutic application.

Hypnotic Activity

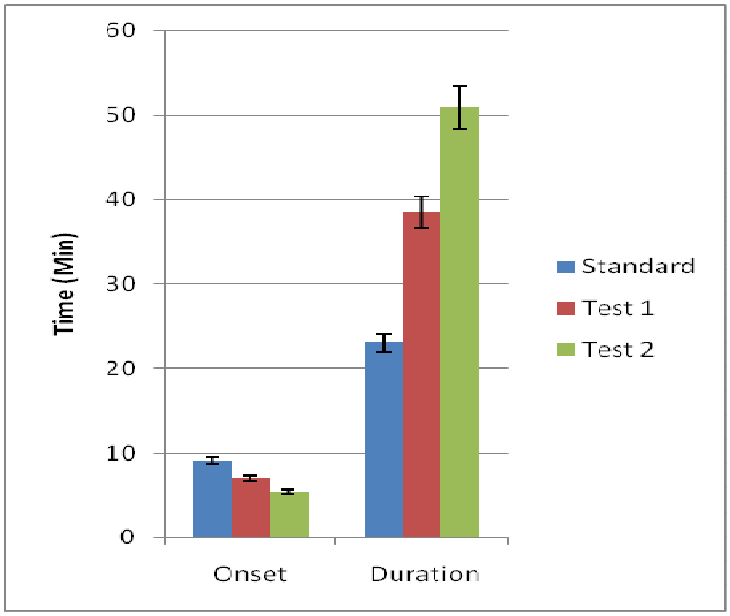
Diazepam-induced sleep in mice: The results for the effect of Arq Gulab on diazepam induced sleep were shown in **Table 4** and depicted in **Figure 2**. The onset of sleep was decreased significantly in the groups treated with the extract when compared with the control group.
The duration of sleep-induced by diazepam increased significantly from 23±0.73 min in the control group to 38.5±1.118 and 50.8±1.22 in the groups treated with the Arq Gulab at the doses 3 and 6 ml/kg, respectively. The 6 ml/kg of the formulation showed greater decreased onset of sleep and longer duration of sleep when compared with the control group that received diazepam only.

Table 4: Effect of Arq Gulab on Diazepam induced sleep in mice.

Group	Treatment	Sleeping Time (Min)	
		Latency time(onset)	Duration
Standard (control)	Saline (10ml/kg) + Diazepam (3mg/kg)	9.08 ± 0.3	23 ± 0.73
Test 1	Arq Gulab (3ml/kg) + Diazepam (3mg/kg)	6.98 ± 0.13*	38.5 ± 1.118*
Test 2	Arq Gulab (6ml/kg) + Diazepam (3mg/kg)	4.53 ± 0.23*	50.8 ± 1.22*

All values are expressed as Mean ± SEM (n=6); experimental groups are compared with control. The data was analyzed by one-way ANOVA followed by Dunnet's multiple comparision test and values are statistically significant at *P<0.01.

Figure 2: Effect of Arq Gulab on onset and duration of sleep in diazepam induced sleep compared to normal saline in mice.



Arq Gulab significantly and dose-dependently reduced the onset and prolonged the duration of sleep-induced by diazepam. By potentiating diazepam-induced sleep, Arq Gulab seems to possess sleep-inducing properties (27, 28). Sedative hypnotic agents act to increase Gamma Amino Butyric Acid (GABA) mediated synaptic inhibition either by directly activating GABA receptors or, more usually, by enhancing the action of GABA on $GABA_A$ receptors.

Benzodiazepines and barbiturates are examples of widely used therapeutic agents that act as positive allosteric modulators at $GABA_A$ receptors (29). Benzodiazepines bind to the gamma-sub-unit of the $GABA_A$ receptor that causes an allosteric (structural) modulation of the receptor results with an increase in $GABA_A$ receptor activity. Diazepam acts selectively on $GABA_A$ receptor, which mediates fast inhibitory synaptic transmission throughout the central nervous system. The ability of the Arq Gulab to potentiate the sedative property of diazepam suggests that it may possibly act by interacting with GABA-mediated synaptic transmission.

Conclusion

Unani medicines are used extensively, but as they lack modern scientific evidence, they are not accepted by conventional medicine practitioners. From the above results, it was concluded that Unani formulation Arq Gulab possesses significant hypnotic activity and antioxidant activity, can be used as easily accessible source of natural antioxidants and it could be used for the treatment of oxidative stress induced disorders. This study provides experimental support for the use of this formulation in folklore medicine for nervous and other disorders. However, the components responsible for the antioxidant activity, hypnotic activity are currently unclear, further studies should be performed to identify the mechanisms underlying the activities observed and also on the isolation and identification of phyto constituents, toxicity and other pharmacological studies to explore its utilization.

References

1) Eisenberg DM, Kessler RC, Foster CNorlock, CECalkins, DRD Delbanco TL. Unconventional medicine in the United States- Prevalence, costs and Pattern of use. N Engl J Med 1993; 328:246-252.
2) Ahmed B, Akthar J. Unani system of medicine. Pharmacognosy Review 2009; 1:210-213.
3) Anupama K, Satyanarayana S, Mukkanti K, Khan KA, Kumar KP. Protective activity of Hab-e-jund a unani formulation against convulsions in mice. Pharmacology online 2009; 3: 724-731.
4) Rawat MS, Rama Shankar. Distribution status of Medicinal Plants conservation in Arunachal Pradesh with special reference to National Medicinal Plants Board. BMEBR 2003; 24 (1-4): 1-11.

5) Mohmad K. "Baiz Kabir", vol-II. New Delhi: Ejaz publishing house, 2001:135.
6) Ara N, Nur H. *In vitro* Antioxidant Activity of Methanolic Leaves & Flowers Extracts of *Lippia Alba*. Research Journal of Medicine & Medical Sciences 2009; 4(1): 107-110.
7) Audipudi AV, Bhaskar VC. Antioxidative and Antimicrobial Activity of Methanol and Chloroform Extracts of *Gmelina Arborea* Roxb. International Journal of Biotechnology and Biochemistry 2010; 6(1):139–144.
8) Bhandari P, Kumar N, Gupta AP, Singh B, Kaul VK. A rapid RP-HPTLC densitometry method for simultaneous determination of major flavonoids in important medicinal plants. Journal of Separation Science 2007; 30(13):2092-2096.
9) Schiber A, Mihalev K, Berardini N, Mollov P, Carle R. Flavonol glycosides from distilled petals of Rosa damascena Mill. Z Naturforsch(C) 2005; 60(5-6):379-84.
10) Watanabe S, Hashimoto I, et al. Isolation and identification of 2-phenylethyl disaccharide glycosides and monoglycosides from rose flowers, and their potential role in scent formation. Biosci Biotechnol Biochem 2001; 65(2):442-5.
11) Boskabady MH, Kiani S, Rakhshandah H. Relaxant effects of Rosa damascena on guinea pig tracheal chains and its possible mechanism(s). J Ethnopharmacol 2006; 106(3):377-82.
12) Basim E, Basim H. Antibacterial activity of Rosa damascena essential oil. Fitoterapia 2003; 74(4):394-6.
13) Biswas NR, Gupta SK, et al. Evaluation of Ophthacare eye drops- a herbal formulation in the management of various ophthalmic disorders. Phytother Res 2001; 15(7):618-20.
14) Mahmood N, Piacente S, et al. The anti-HIV activity and mechanisms of action of pure compounds isolated from Rosa damascena. Biochem Biophys Res Commun 1996; 229(1):73-9.
15) Baskar B, Rajeswari V, Satish Kumar T. Invitro antioxidant studies in leaves of *Annona* species. Indian J Exp Biol 2007; 45:480 - 485.
16) Kenjale RD, Shah RK, Sathaye SS. Antistress and antioxidant effects of *chlorophytum borivillianum*. Indian J Exp Biol 2007; 45:974 - 979.
17) Sharma SK, Gupta KV. Free radical scavenging activity of *Ficus racemosa* roots. Indian Journal of Pharmaceutical Education & Research 2007; 41:394-396.

18) Sreejayan N, Rao MNA. Nitric oxide scavenging activity by curcuminoids. Journal of Pharmacy and Pharmacology 1997; 47:105.
19) Oyaizu M. Studies on product of browning reaction preparation from glucose amine. Japanese Journal of Nutrition 1986; 44:307-09.
20) Anuradha CV, Kannapan S, Lakshmi Devi SL. Evaluation of invitro antioxidant activity of Indian bay leaf, *Cinnamomum tamala* (Buch. - Ham.) T.Nees & Ebern using rat brain synaptosomes as model system. Indian J Exp Biol 2007; 45:378–384.
21) Beretz A, Haag-Berrurie M, Anton R. Choix de méthodes pharmacologiques pour l'étude des activites de l'aubépine. Plantes Medicinales et Phytotherapie 1978; 4: 305-314.
22) Rakotonirina, S.V., Bum E, Rakotonirina A, Bopelet M. Sedative properties of the decoction of the rhizome of Cyperus articularis. Fitoterapia 2001; 72:22-29.
23) Miya, T.S., Holck HGL, Yu GKW, Spratto GR. Laboratory guide in pharmacology. Minneapolis MN: Burgess Publishing Company, 1973:44-46.
24) Fujimori H. Potentiation of barbital hypnosis as an evaluation method of central nervous system depressant. Psychopharmacology 1965; 7:374-397.
25) Soulimani R, Younos C, et al. Behavioral and pharmaco-toxicological study of Papaver rhoeas L.in mice. J Ethnopharmacol 2001; 74:265-274.
26) Duncan RC, Knapp RG, Miller MC. Test of hypothesis in population means. In: Introductory Biostatistics for the health sciences. NY: John Wiley and Sons Inc, 1977:71-96.
27) Guillemain, J. And Tetau M. Contribution à l'étude d'un "tranquillisant végétal" Tilia tomentosa Bourgeons. Cahiers de Biothérapie 1980; 68:1-8.
28) Rakotonirina, S.V., Bum E, Rakotonirina A, Bopelet M. Sedative properties of the decoction of the rhizome of Cyperus articularis. Fitoterapia 2001; 72:22-29.
29) Johnston, G.A.R. GABAA Receptor Channel Pharmacology. Curr Pharm Des 2005; 11:1867-1885.