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Study Of Different Parameters On Formulation, Phytochemical & Pharmacological Profile Of Liquorice Wine

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Abstract:

Traditional Indian medicine system has a large variety of formulations in different dosage forms which were prepared and dispensed as per patient need. Out of these the arishtas and asavas are self-generating alcoholic herbal formulations but due to increasing popularity of allopathic system of medicine these are at a moderate stage and need to evolve. As against these, wine a popular European anti-oxidant drink has gained popularity all over. Our effort is to promote the traditional medicine system by extemporizing the dosage form to a more palatable while maintaining identical efficacy. The popular variety of wine is from grapes albeit it can be made from other fruits or herbs. In the present study we propose the formulation, evaluation of a wine from *Glycyrrhiza glabra* Linn. root and rhizomes. The study involves comparison of its efficacy over liquorice syrup available in traditional medicine system. Liquorice or *Glycyrrhiza glabra* Linn., has ethnopharmacological values due to the presence of glycyrrhizin, 18β-glycyrrhetinic acid, glabrin A, glabrin B, isoflavones, etc and is of great therapeutic value in a number of diseases. An effort was made to combine the medicinal effect of both drugs which would prove to be therapeutically beneficial and would augment the process of healing.

Introduction

Herbal formulations including traditional Indian medicine have reached extensive acceptability as therapeutic agent for several diseases. Arishtas and Asava are self-generating alcoholic herbal formulations of traditional Ayurvedic system. These alcoholic remedies are prepared by fermenting the herbal juice and its decoction by addition of sugar. The study of different categories of herbs and formulations along with fermented products such as the fermented decoctions or the arishtas and the fermented infusions such as Asava. Looking through other traditional medicinal systems prevalent in the world Wine a popular drink as

anti-oxidant and extensively used in European countries. Not only grape wine but even other medicated wines are popular in EU.

Glycyrrhiza glabra Linn., has ethno pharmacological value and is used in Ayurveda, siddha and unnani systems. The phytochemical constituent glycyrrhizin, 18β-glycyrrhetinic acid, glabrin A, glabrin B, isoflavones etc, have validated the use of plant since ancient times as antioxidant, antibacterial, antiviral, anti-inflammatory and antidiabetic action. An additional agent added are the Woodfordia Fruticosa flowers which have been reported to possess high therapeutic efficacy. The phytochemicals present include flavonoids, tannins, glycosides, anthraquinone and polyphenols. The extract of flowers and leaves is found active as hepatoprotective, antimicrobial, antioxidant, cardioprotective, immunomodulatory, antiulcer, antifertility and anti-tumor activities. Addition of the extract increases the value and scope of medicated wine. [1,2,3]

Regarding evaluation, the WHO assessment guidelines specify quality along with safety and efficiency of herbal medicines as pre-requisites for global harmonization as utmost important. Hence, standardization is an obligatory aspect for establishing the quality and/or efficiency of ayurvedic preparation or any other multiple ingredient herbal formulation. Hence an endeavor for evaluation of wine preparation for quality and efficacy has been attempted. The objective of the present study can be summarized as Formulation development of Liquorice wine, its evaluation and comparison of in-house formulations of liquorice wine with liquorice syrup. And also biological and analytical study for liquorice formulations.

Material and Methods

Collection and identification of the plant material: Fresh root and rhizomes of Glycyrrihiza glabra and flowers of Woodfordia fructicosa (dhataki) were collected and identified and authenticated to confirm whether drug is pure or adulterated. Fresh plant material was washed with tap water, air dried and then homogenized to fine powder and stored in airtight bottles.

Raw material selection and analysis was done per WHO guidelines. Preliminary Phytochemical Screening involved[5] ethanolic extract of fresh Glycyrrihiza glabra was subjected to preliminary phytochemical screening for the detection of various plant constituents as per table 1.

Microbial load for wine and syrup: Total bacterial and total fungal counts as well as specific count for Escherichia coli, Salmonella typhi and Pseudomonas aeruginosa for crude powder and ethanol extract of Glycyrrihiza glabra (Leaf + stem) was carried out using reported methods.[6]

Table 1: Physico-chemical characterization of Glycyrrhiza glabra powder

Sr. No.	Parameters	Standard Range	Observed	Compliance
1.	Loss on drying (105°C for 6 hoursrs)	≤ 10.0%	7.62%	Complied
2.	Total ash	≤ 7%	6.90 %	Complied
3.	Water-soluble ash	≥ 20%	27.56%	Complied

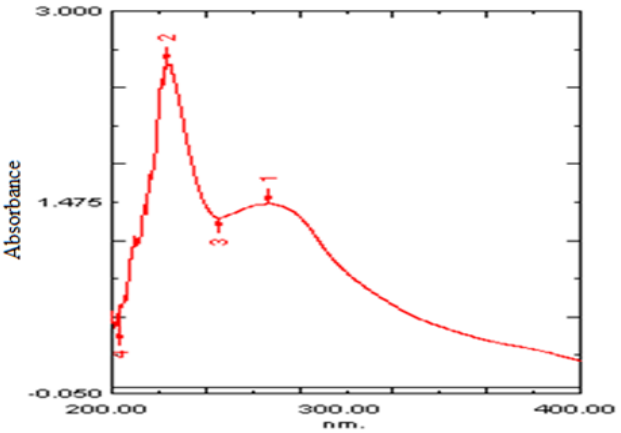
4.	Acid-insoluble ash	≤ 2%	1.80 %	Complied
5.	Alcohol-soluble ash	≥ 25%	31.46%	Complied

Table 2: Extractive value of Glycyrrhiza glabra powder in different solvents

Sr. No.	Solvent	Extractive value
1.	Water	12.57%
2.	Ethanol	10.42%
3.	Methanol	10.05%
4.	Petroleum ether	3.23%

Table 3: Phytochemical evaluation of ethanolic extract of powdered liquorice

Sr. No.	Plant constituents	Test/reagent	Observation
1	Alkaloids	Wagner's reagent test Hager's reagent test	Absent Absent
2	Flavonoids	Shinoda's test Lead acetate test H_2SO_4 test	Present Present Present
3	Tannin	Braymer's test Lead acetate test	Absent Absent
4	Saponin	Foam test	Present
5	Carbohydrates	Molisch test	Present
6	Reducing sugar	Fehling's test	Present
7	Quinones	HCl test	Absent
8	Terpenoids	Liebermann-Burchard test	Absent
9	Sterols	H_2SO_4 test Liebermann-Burchard test	Absent Absent
10	Phenols	Ferric chloride test Libermann's test	Present Present
11	Anthroquinone	Borntrager's test	Absent
12	Anthocyanins	NaOH test	Absent
13	Proteins	Ninhydrin (aqueous) test Ninhydrin (acetone) test	Absent Absent



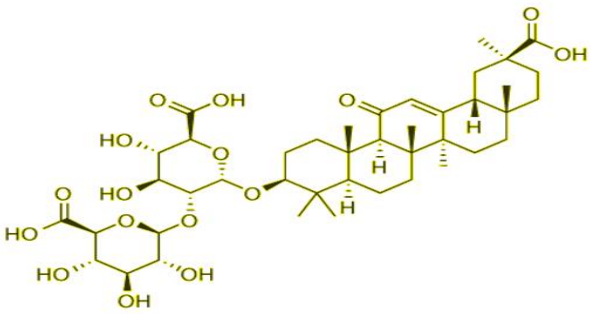


Fig No- 1: UV spectrum of Liquorice extract **Fig No. 2:** Structure of Glycyrrhizic acid

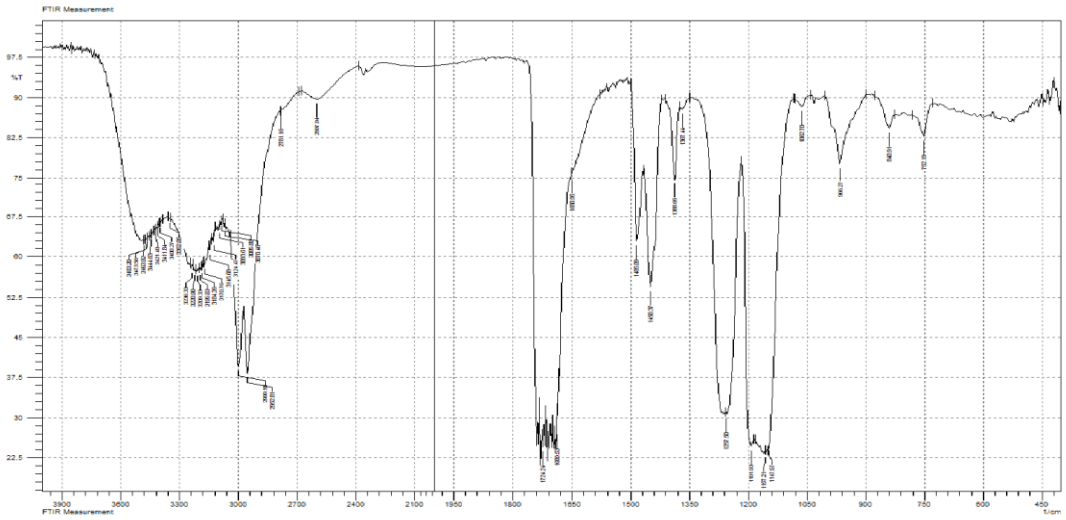


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Figure 3: FT-IR spectra of isolated Glycyrrhizic acid

Formulation Studies:

Evaluation of wine formulation: the wine was evaluated on appearance, aroma, taste, pH, volatile acidity, presence of free sulfur di-oxide, titrable acid, etc. This gives some basic knowledge about evaluation of wine.

Appearance

Total Soluble Solids (TSS): It was determined by Abbey's Refractometer and is expressed as °Brix (°Brix = 1 gm sucrose /100 gm juice).

pH: The pH of wine sample was determined using pH meter.

Titrable acidity (TA): Both parameters were determined by performing acid base titration[7].

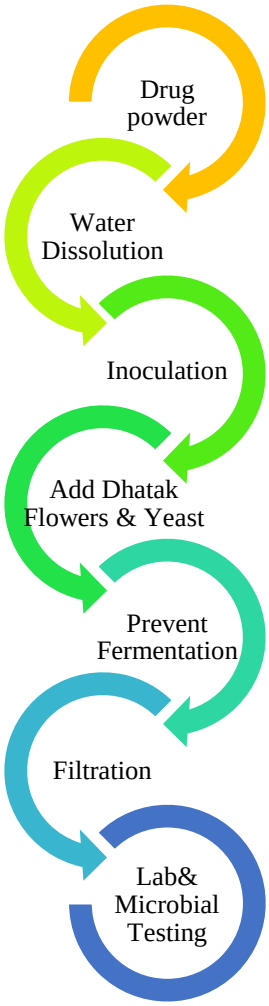

Figure 4: Flow chart for syrup formulation

Free-SO_2: It was determined by using Aeration Oxidation method [7] .

Reducing sugar (RS): Gold Coast method was used to determine RS[8] .

Volatile acidity (VA): It was determined by steam distillation method.

Total Phenolic Content (TPC): It was measured using Folin ciocalteu assay[9,10].

Total Flavonoid Content (TFC): Determined using Aluminium chloride colorimetric assay[9,10]. was measured using The DPPH free radical scavenging assay was carried out to measure antioxidant activity[11,12].

GC Analysis: Thermo Scientific Chemito GC-1000 (detector: FID Column: SGP BP624 -L:30m, T: 3mm) was used for GC analysis. Sample size: 0.5µL, Carrier Gas: Nitrogen, Flow rate: 3ml/min (constant flow rate) Run time: 10-15 min.
UV method development for quantization of glycyrrhizic acid as per ICH guidelines

Development of HPLC method for estimation of GA as per ICH guidelines: Analytical Method Development was done for quantitative estimation of glycyrrhizic acid on HPLC. Mobile phase: acetonitrile: water: 70:30 and 0.01% glacial acetic acid. Sample size: 0.5µL, Flow rate: 3ml/min (constant flow rate) Run time: 10-15 min.

FT-IR spectrometry: A drop of wine sample was squeezed in between the two sodium chloride plates (0.1-0.3mm thin), it's infrared spectra was recorded at 4000cm^{-1} to 400cm^{-1} using Fourier transform infra-red spectrophotometer (Model . 8400S Shimadzu)

In-vitro anti-oxidant activity by DPPH radical scavenging assay: DPPH radical scavenging activity of extract, based on the scavenging activity of the stable 2, 2-diphenyl-2-picryl hydrazyl (DPPH) free radical, and absorbance measure at 517 nm. The inhibition curves were prepared and IC_{50} values were obtained by Probit analysis.
Pharmacological study was carried out by anxiolytic activity elevated four plus maze method.

RESULT AND DISCUSSION

Wine a popular drink as anti-oxidant and extensively used in European countries. Not only grape wine but even other medicated wines are popular in EU. The proposed project deals with the formulation, evaluation and efficacy testing of Glycrrhiza Glabra wine over Glycyrrhiza glabra syrup. The popular Liquorice medicinal remedies include treatment of inflammation, cough suppressant and increased sputum secretion in air passages. It is also found to be active against bacteria, ulcer, allergy and also suppressing spasm. In Ayurveda the Liquorice syrup is commonly used as expectorant. In present study, the liquorice wine was formulated using different experimental conditions like time duration for fermentation, varying concentration of active principle, different fermentation temperature and performed in different fermenters.[13,14]

The fresh root and rhizomes of Glycyrrhiza glabra and flowers of Woodfordia fructicosa (dhataki) were collected, identified and authenticated. The collected fresh plant parts were washed with water, dried in shade and then blended into uniform fine powder. To prevent damage from environment they were stored in a sealed container for further use. The physico-chemical characterization of raw material was carried out and is reported in table no. 1.

The moisture content of the drug was found to be moderate hence it could discourage bacteria, fungi or yeast growth. Similarly, the ash value and acid insoluble ash value determination were found to be within limits for herbal drugs. The total ash indicative of presence or absence of foreign inorganic matter such as metallic salts and/or silica also meet the standard. Comparative extraction study in different solvents is mentioned in table no. 2. The powdered liquorice drug was found to comply for all parameters of physicochemical properties as per WHO guidelines.

The ethanolic extract of liquorice was evaluated for various phytoconstituents. Phytochemical analysis of liquorice shows the presence of flavonoids, saponins, phenols, carbohydrates, and therapeutically active phyto-constituent. These results are summarized in table no. 3.

Isolation of Glycyrrhizic acid: The dried liquorice slices were extracted with aqueous ammonia solution (0.5%, v/v) by sonication. The ammonia extract was then passed through silica column for purification of extracted Glycyrrhizic acid. Further the column passed extract was dried, and used for authentication by thin layer chromatography, melting point, uv-visible and IR spectroscopy.

Identification of Glycyrrhizic acid: The Rf for glycyrrhizic acid in extract was observed at 0.47 as against reported Rf 0.45. The melting point of isolated compound was observed as 182-184°C. For uv-spectrometric study an appropriate quantity of ethanolic liquorice extract was dissolved in methanol and UV spectrum recorded in the range of 200 to 400 cm-1 (figure 1). The λmax observed was 254nm which indicated the presence of glycyrrhizic acid. Further analysis of major functional groups was done by FTIR in the range of 500-4000 cm^{-1}, which confirmed presence of Glycyrrhizic acid in the extract. The FTIR spectra indicates the presence of hydroxyl, carbonyl, sugar moiety, ether and benzene support the confirmation of Glycyrrhizic acid (figure 2 and 3). 3150-3480cm-1, (b)(s), O-H str indicative of sugar moiety (glycoside); 2900-3300cm-1 (s), -OH str –COOH indicative of sugar moiety (glycoside), 2950-2999cm^{-1}, C-H stretch of CH2,1724 cm^{-1} (s), -CH2= CH2, (alkene), 1470–1450 cm^{-1} (m), -C–H bend of CH2 (alkanes); 1170-1250 cm^{-1},-C-O-C-, (ether); 752, 840,966 cm^{-1}, bend, out of plane, (aliphatic ring substitution).

Formulation Studies: The procedure followed for preparation of Liquorice wine is depicted in figure 4. The four parameters considered during the process were temperature, fermenter, concentration of ingredients and duration. Further on, above produced wine was used for checking its evaluations, microbial load, analytical method validation and checking its pharmacological activity.

Formulation of Liquorice Wine: Based on the parameters of variation following eight formulations were prepared and studied for the alcohol content and observations were recorded in table number 4. It was observed that the alcohol content in the preparation made using dhakati's flowers was greater as compared to that prepared using yeast. Changing concentration of liquorice powder caused the alcohol content to vary. Also the amount of self-generated alcohol was increased with concentration of liquorice powder. In case of jaggery, the self-generating alcohol content was found to be greater however productions of undesirable components like n-butanol, propanol, etc. were obtained which were absent in non-jaggery formulation. Lastly the self-generating alcohol content was increased at room temperature (24-25°C) as compared to refrigerator temperature (2-4°C).

Table no. 4- Formulation and fermentation conditions

Sr. No.	Formulation Name	Sample	% Alcohol
1	A	99.99% absolute Ethyl alcohol	100
2	B_1	100ml water + 10gm Liquorice powder + Yeast	7.71
3	B_2	100ml water+10 gm Liquorice powder+ Flowers	26.51

4	C	25ml water + 2gm Liquorice powder + Yeast	4.22
5	D	25ml water + 3gm Liquorice powder + Yeast	6.57
6	E	25ml water + 4gm Liquorice powder + Yeast	6.90
7	G	100ml water + 10gm Liquorice powder + Yeast + 5gm Jaggery	28.8
8	H_1	150ml water +10gm Liquorice powder + 10gm Flowers (Refrigerator)	8.45
9	H_2	150ml water +10gm Liquorice powder + 10gm Flowers (Room temp)	9.74

Physicochemical evaluation of wine formulation B1: We considered B1 wine formulation for further study as the wine was free of toxic constituents like n-butanol, n-propranol, etc. the results are summarized in table number 5

Table No 5: Physicochemical parameters for wine evaluation:

Sr. No.	Parameter	Standard Range	Observed	Compliance
1.	pH	2.9 to 4.0	3.6	Comply
2.	Residual sugar	1-3g/l	1.4 g/l	Comply
3.	Soluble solid	≤ 10%	9.6%	Comply
4.	Refractive index	7 to 10 ^{0}Brix	8-9 ^{0}Brix	Comply
5.	Volatile acidity	≥ 25%	0.117 g/100ml	Comply
6.	Tartaric acidity	≤ 0.05 mg/kg	0.0237 mg/kg	Comply
7.	Free sulfur dioxide	≥ 0.825 mg/l	1.3 mg/l	Comply

It was found that wine formulation B1 complies for all the conditions for physicochemical testing.

Evaluation of syrup formulation: The syrup formulation was evaluated for its preliminary characteristics like organoleptic properties including color, odour, taste, pH, specific gravity and viscosity.

Formulation and Evaluation of Liquorice Syrup: The Liquorice syrup was formulated as per B.P 2011 procedure with sugar content of 66.7%. The syrup was evaluated for physicochemical parameters as per B.P. The formulation was colorless, sweet liquid having almost neutral pH 6.8, specific gravity 1.216 and viscosity 54.63centipose. The density and viscosity of formulation was due 66.7% sugar content. Further on, syrup was checked for its pharmacological activity.

Evaluation:

Analytical Methods: UV method development for quantization of glycyrrhizic acid.

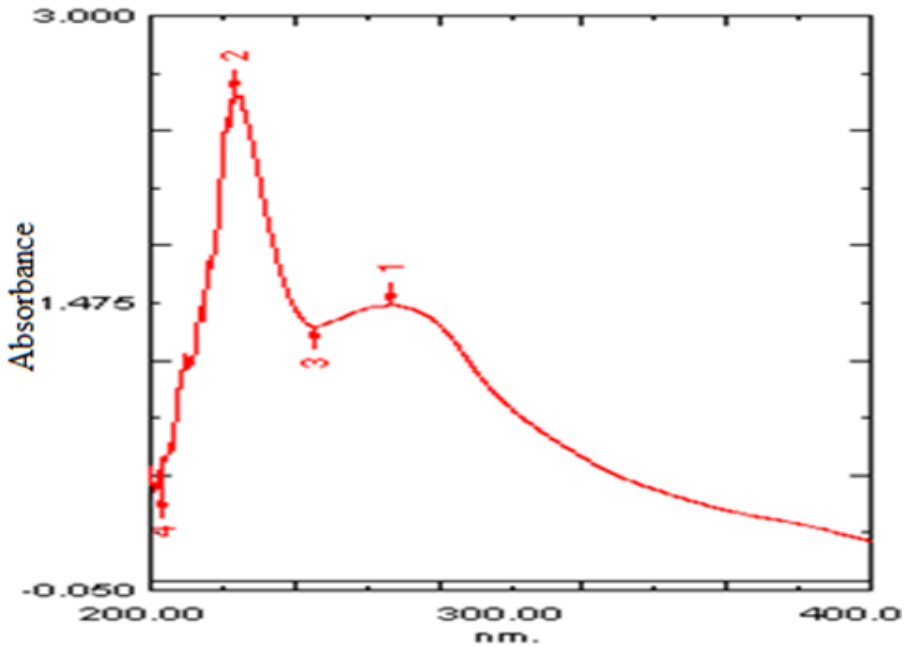

Figure 5: UV spectrum of Glycyrrhizic acid (256nm)

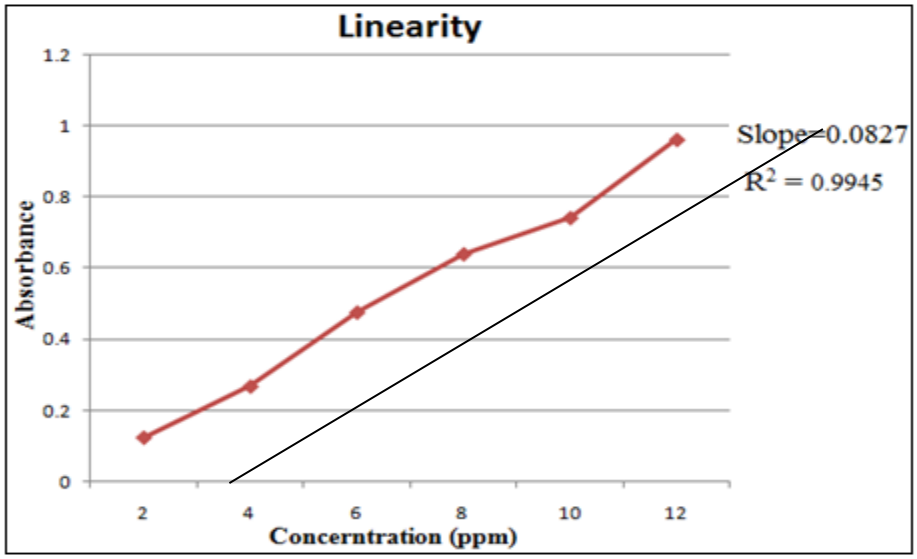

Figure 6: Calibration curve of Glycyrrhizic acid by UV method.

R^2 = 0.9945; Slope=0.0827, the Lambert – Beer's law was obeyed at concentration range of 2-12 ppm with regression coefficient (R^2) of 0.9945, LOD and LOQ were found to be 0.99 and 3.022 respectively. For precision standard deviation: 0.0025 and relative standard deviation: 0.32%. The standard error between the absorbance of two solvent systems and two instruments was found to be 0.002 and 0.0018 respectively. The Standard Error between the two observations of intraday and interday precision was found to be 0.00218 and 0.00222 respectively. The recovery study was performed at low, medium and high level with recovery in the range of 95.98 to 97.34%. The quantization of Glycyrrhizic acid (GA) was analyzed by UV method. Standard λ_{max} for GA is 256nm. The calibration curve was plotted using concentration against absorbance. The curve obtained was linear within the concentration range of 4-12

µg/ml. The correlation co-efficient value for the calibration graph was found to be 0.9945. The precision of the method was confirmed by Intraday and Inter day analysis. The method gave precise results during intra-day and inter-day precision. LOD and LOQ of the method were found to be less than 1 µg/ml i.e. 0.99µg/ml and 3.022 µg/ml respectively. The accuracy of the method was performed by recovery studies. The percentage recovery was found to be in the range of 95.98- 97.34%. This indicates that this method is very accurate. Finger printing by HPLC methods for quantization of glycyrrhizic acid . HPLC method development for quantization of glycyrrhizic acid was found in wine formulation by HLPC using acetonirile:water (70:30) with 0.01% glacial acetic acid as solvent system. The retention time of glycyrrhizic acid present in extract and wine was found to be 2.26 min and 2.41 min respectively.

Quantitative phytochemical screening included total phenol determination in which gallic acid was determined by uv-spectroscopy method was developed and validated. Total Phenolic Content was calculated as a gallic acid equivalent and was found to be 3.33 **±** 0.072 mg/ml of wine. The relatively good amount of Total Phenolic Content in wine formulation indicates the good anti-oxidant property of the formulation. Total flavonoid content was calculated as a catechin equivalent and was found to be 1.032**±** 0.039mg/ml of wine. The flavonoid compound is responsible for anti-oxidant activity.

In-vitro anti-oxidant activity on wine and syrup formulation:

DPPH radical scavenging activity:

Ascorbic acid and DPPH was studied and observations noted in

Table No- 6: Observation table for Ascorbic acid and DPPH

Sr. No.	Conc (ppm)	Absorbance	% Inhibition at 512nm
1	0	0.0	0
2	10	0.130	87.14
3	20	0.0904	91.05
4	30	0.0880	91.29
5	40	0.0779	92.29
6	50	0.0660	93.47
7	60	0.0530	94.75
8	70	0.0480	95.25
9	80	0.0389	96.15
10	90	0.0331	96.72
11	100	0.0250	97.52

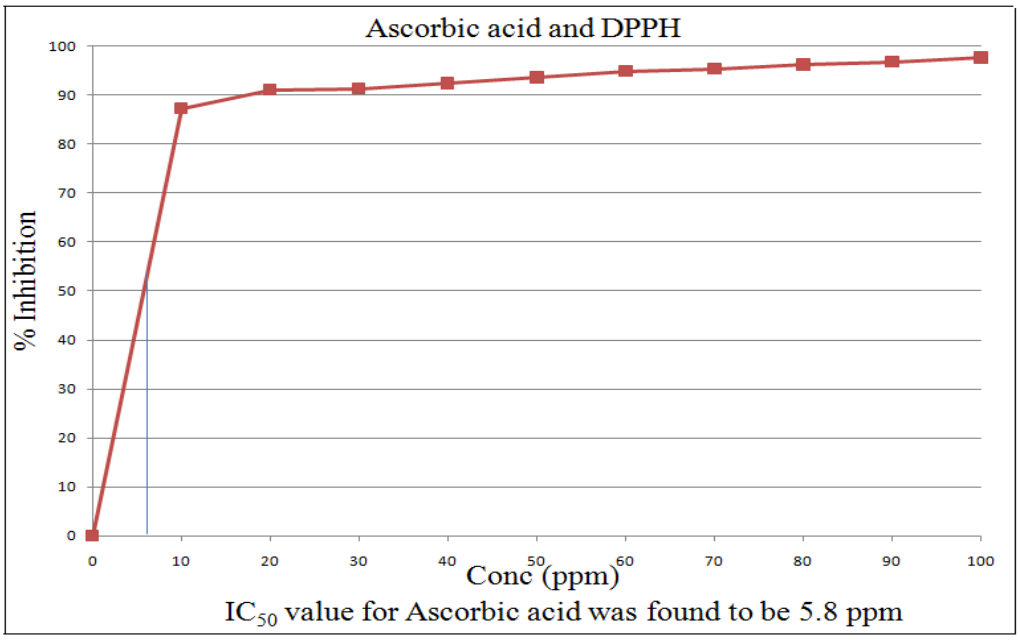

Figure 7: Graph of % inhibition of Ascorbic acid and DPPH

ii) Wine and DPPH

Table No- 7: Observation table for Wine and DPPH

Sr. No.	Conc (ppm)	Absorbance	% Inhibition at 512nm
1	0	0.0	0
2	10	0.430	57.46
3	20	0.389	61.51
4	30	0.280	72.3
5	40	0.220	78.23
6	50	0.189	81.3
7	60	0.170	83.18
8	70	0.150	85.16
9	80	0.110	89.11
10	90	0.099	90.08
11	100	0.089	92.08

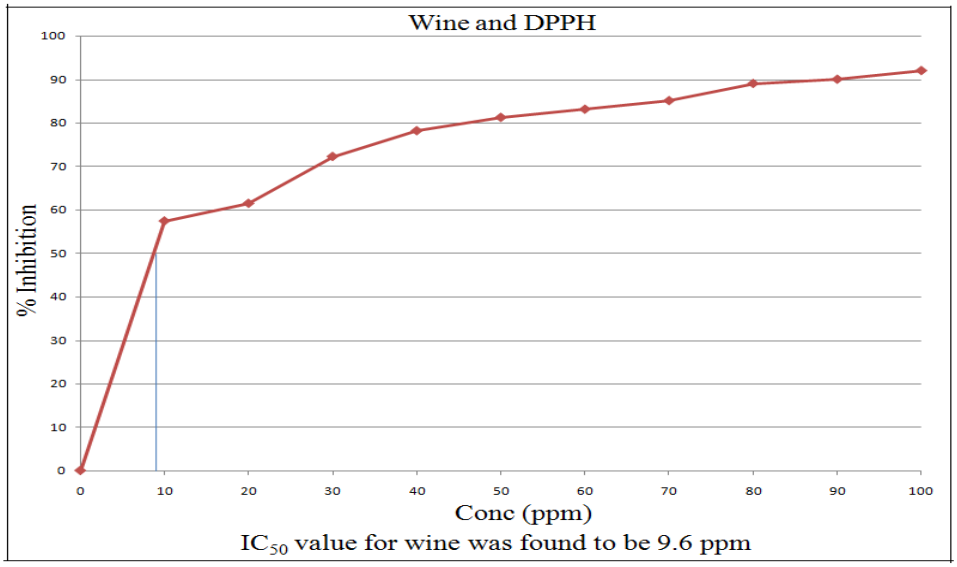

Figure 7: Graph of % inhibition of Wine and DPPH

The liquorice wine shows good anti-oxidant activity with 9.6 ppm as IC_{50} value when compared to ascorbic acid as indicated in figure 7.

Pharmacological study:

Anxiolytic activity elevated four plus maze method was determined

Table 8: Effect on time spent by mice in open arm and closed arm of elevated plus maze

Treatment (Dose: mg/kg)	Time spent in open arm	Time spent in close arm
Vehicle	67.5±2.97	84.7±2.51
Diazepam (1)	195.7±2.35*#	98.6±3.59*#
Liquorice Syrup (10)	145.3±4.56*	129.5±0.51*
Glycyrrhzic acid (10)	163.1±3.25*#	112.8±1.95*#
Liquorice wine (10)	189.7±2.35*#	105.5±2.56*#

n=5; *p <0.001 significantly compared with vehicle followed by One- Way ANOVA by Dunnett's test; # p < 0.05, Diazepam, Glycyrrhzic acid, Liquorice wine significantly compared with each other by Bonnferonni test

Table 9: Effect on number of entries in open arm and closed arm of the elevated plus maze in mice

Treatment (Dose: mg/kg)	Number of entries in open arm	Number of entries in close arm
Vehicle	5.7±0.23	7.4±0.56
Diazepam (1)	9.5±0.46*#	2.3±0.46*#
Liquorice Syrup (10)	7.6±0.56*	8.7±0.40*
Glycyrrhzic acid (10)	8.0±0.23*	6.2±0.25*
Liquorice wine (10)	8.6±0.78*#	5.5±0.45*#

n=5; * p<0.001, significantly compared with vehicle followed by One way ANOVA, by Dunnett's test; # (p<0.001) Diazepam and Red wine significantly compared with each other by Bonnferonni test.

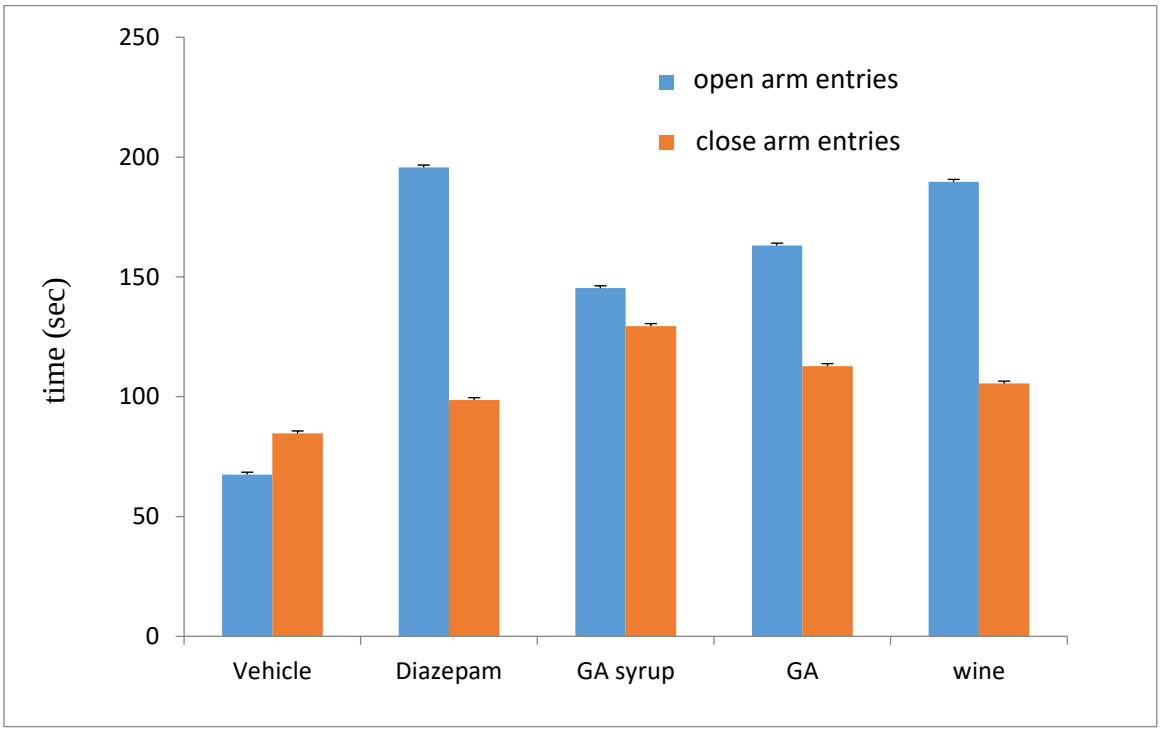

Figure 8: Effect of various treatments on time spent in open and closed arm

The in vivo Anxiolytic activity (Elevated plus maze method) for wine sample was performed, which showed good anxiolytic effect and significantly followed by One- Way ANOVA when compared with Diazepam. A comparison between liquorice wine and Glycyrrhizic acid showed enhanced number of entries in open arm when compare with vehicle. Comparing liquorice wine with vehicle, wine shows potentiate Anxiolytic activity as observed in figure 8.

CONCLUSION

The liquorice wine prepared was found to be more effective as it shows potentiate anxiolytic activity over liquorice syrup. The liquorice wine shows good anti-oxidant activity with 9.6 ppm as IC_{50} value when compared to ascorbic acid. Wine formulation hence was effective over syrup.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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