Research Article
CODEN (USA): IJPLCP

[Joseph et al., 3(6): June, 2012]
ISSN: 0976-7126

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES

Enhancement of antioxidant profile of Japanese cherry (*Muntingia calabura* Linn.) by alcoholic fermentation

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Abstract

Japanese cherry (*Muntingia calabura*) with supplements to promote yeast growth was subjected to alcoholic fermentation for 28 days. A clear fluid with 10 percent alcohol (v/v) was obtained. It contained 1.68 mg per ml total phenolics (as gallic acid equivalent) and 1.3 mg per ml of reducing power factor (as tannic acid equivalent) which were about 2 fold and 1.6 fold increases respectively over the unfermented must. The fermented fluid offered total protection to salmon milt DNA against experimentally induced damage *in vitro* in comparison to only partial protection by the unfermented fluid. The fermented fluid also inhibited the growth of *Escherichia coli, Salmonella typhi* and *Staphylococcous aureus* cultures and caused the inactivation of *E.coli* in biofilm morphology. The wine of Japanese cherry was found to be comparable in acceptability to a market sample of traditional white wine.

Key-Words: Cherry wine; Antioxidant; DNA damage protection; *E-coli* biofilm destruction; Hedonic rating

Introduction

Intensive search is on for antioxidant-rich plant products with the premise that they can be used in formulations and designed-diets for general health improvement and also for treatment of several human degerative diseases (Pokorny, 1991). Examination of fruits, seeds, leaves, bark etc. has led to the isolation of a wide range of water-soluble and lipid miscible compounds such as phenolics, terpenoids, curcuminoids, tannins, xanthans, flavonoids, carotenoids, etc., with antioxidant properties(Larson,1998; Duthie and Crozier,2000; Jayaprakasha et al, 2002). Much of the data suggestive to the beneficial effects of these phytochemicals were obtained from *in vitro* studies and from epidemiological data which indicated correlation of lowered prevalence of cardio-vascular diseases, cancers and slowing down of ageing with intake of antioxidant-rich food items in humans (Sauthon,1998). More tangible prophylactic or therapeutic applications for natural antioxidants and phytochemicals are however, yet to be conceived. The main difficulty is in finding a suitable form or route by which such phytochemicals can be administered.

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Apart from consuming the selected plant–based foods (fresh fruits, vegetables etc), fruit wines could offer an attractive and effective means to administer health promoting phytochemicals to a greater advantage. The classical red wines made from grapes (*Vitis* spp.) are attributed with beneficial health-promoting effects owing to their flavonoid content (Crozier,2006; de Lange,2007). There are several other fruits which are yet to be explored to choose the right ones which have the best health promoting attributes and more importantly, for suitability to produce wine economically and with good acceptability.

In the present work Japanese cherry (*Muntingia calabura*) was chosen to examine its potential for development of a wine. It is one of the less-known and less popular fruits. It is sold in Mexican markets as edible fruit. It is used in fruit pies and made into jams. Its leaf infusion is consumed as tea-like drink (Corner, 1997). It is popular in several South American countries. Some *in vitro* studies have indicated the presence of antioxidants and possible anti-hypertensive and anti-cancer factors in the fruits, leaves and roots of this plant (Shih et al, 2006, Kaneda et al, 1990, 1991; Nshimo et al, 1993, Chen et al, 2005). Apparently there is no published work on attempts to prepare wine from Japanese cherry and evaluate its attributes and acceptability. The present work was therefore aimed at preparation of wine from Japanese cherry and examine its suitability for development as a health drink.

Material and Methods

Reagent and Chemicals

All chemicals used were of guaranteed reagent grade. Gallic acid, tannic acid, citrate and ascorbic acid were procured from Rolex, Mumbai. Folin-Ciocalteu reagent and NaH_2PO_4 $2H_2O$ were from SD Fine chem Ltd., Mumbai. Sodium carbonate and ferric chloride were from Loba Chemi, Mumbai. $Na_2HPO_42H_2O$, ferrous sulphate, ammonium chloride and potassium metabisulfite were from Merck, Mumbai. Trichloro acetic acid was from Finar, Ahmedabad. Potassium ferricyanide was from Ranbaxy, Mumbai. Dehydrated media for nutrient broth, nutrient agar and tryptic soya broth and Salmon milt DNA were obtained from Hi-media, Mumbai. A popular brand of grape white wine was procured from local market in Mysore, India.

Cultures

E.coli 1610 and *S.typhi* 98 were obtained from Microbial Type Culture Collection of Institute of Microbial Technology, Chandigarh, India. *Staph aureus* MRSA was kindly gifted by the pathology department of Vikram Hospital , Mysore , India. Baker's yeast (AM Mauri)was obtained from local market in Mysore, India.

Preparation of wine

Well-ripened fruits of Japanese cherry (*Muntingia calabura*) were handpicked from the trees, washed with tap water and stored in refrigerator until taken up for further processing.

0.5 kg of fruits was partially mashed using mortar and pestle and suspended in 2.0 liter of water. The slurry was supplemented with 0.5 kg of cane sugar, 10.0 g of ammonium chloride and 0.58 g of potassium metabisulfite .Yeast inoculum was prepared by adding 500 mg of dry baker's yeast to 50 g of the above fruit mash with supplements and allowing growth for 16 hrs. After adding the developed inoculum to the bulk of the fruit mash, fermentation was allowed for 28 days. Small aliquots were drawn once in 7 days for analysis. At the end of 28 days the fermented material was filtered by suction through a pad of washed filter paper pulp. This yielded a clear pale brown colored fluid.

Alcohol in the wine was estimated by distillation and hydrometry according to method of Amerine and Ough (1974). Total reducing sugar was estimated by titrimetry as per Indian Standards Institution (1984). Total acidity was estimated titrimetrically as per Ranganna (2005).

Estimation of total phenolics

Total phenolics content was determined by using Folin-Ciocalteu (FC) reagent according to Spanos and Wrolstad (1990) as adapted by Zahin et al (2001) using gallic acid as the standard. 1 ml of 50 fold diluted sample (diluted with distilled water) was mixed with 2.5 ml of 10 % FC reagent and 2 ml of 7.5 % sodium carbonate solution. The resulting mixture was incubated at 45°C for 15 mins. Absorbance was measured at 765 nm using a UV-visible spectrophotometer. Results were expressed as gallic acid equivalent in mg/ml of the fermented sample.

Reducing power assay

Total reducing power was determined according to the method of Oyaizu (Oyaizu 1986) using tannic acid as the standard. 20 µl of 10 fold diluted sample was mixed with 460 µl of 20 mM phosphate buffer pH 6.6, 500 µl of 1 % potassium ferricyanide and made upto 1000 µl with distilled water. The reaction mixture was incubated at 50 °C for 20 mins and cooled to room temperature. 500 µl of 10 % trichloro acetic acid was added and volume made upto 3ml with distilled water. Finally 300 µl of 0.1 % ferric chloride was added and absorbance measured at 700 nm in spectrophotometer. Results were expressed as tannic acid equivalent in mg/ml of fermented sample.

DNA Protection assay

DNA protection assay was an *in vitro* technique used to measure inhibition of DNA damage induced by ferrous sulfate and ascorbic acid (Halliwell & Gutteridge 1990). DNA damage was monitored by mixing of 20 µl of 1.25 mg/ml salmon milt DNA with 30 µl of 20 mM phosphate buffer pH 6.6, 20 µl of 1mM ferrous sulfate, 20 µl of 10 mM ascorbic acid and 10 µl of fermented sample. The reaction mixture was incubated at 37°C for 4 hrs in water bath. The volume was made upto 3 ml with phosphate buffer and absorbance measured at 260 nm in a spectrophotometer.

Antimicrobial activity tests

The clear fermented fluid was concentrated to one tenth the volume over a water bath and taken up for antimicrobial activity tests in broth-culture and by disc-diffusion methods. Standard *E.coli, S.typhi and Staph.aureus* were employed as test bacterial cultures.

Broth culture

Different volumes of fermented fluid processed as above ranging from 0 to 500 µl were added to 2 ml of sterilized nutrient broth. Volume was made upto 2.5 ml with sterile distilled water. The tubes were inoculated with 10 µl of 16 hrs old bacterial cultures (10^7 cfu /ml) and incubated at 37° C for 24 hr and bacterial growth was recorded as positive and negative to turbidity development. The test was performed in duplicate for each microbial culture.

Disc diffusion method

Sterilized Whatman #1 filter paper discs of 10mm dia were impregnated with different volumes of the processed fermented fluid and allowed to dry in a

desiccator. The discs were then placed on nutrient agar previously surface-spread (1 h earlier) with 16 hrs grown bacterial cultures of sufficient cfu to produce a lawn of growth in Petri dishes. The dishes were kept in incubator maintained at 37ºC for 24 hrs. The diameter of clearance zones (Inhibition zones) were measured in mm.

***E.coli* biofilm sensitivity to fermented fluid**

Development of *E.coli* biofilm

Biofilm of *E.coli* was developed on surface of plastic (HDPE) coupons according to the method of Frank and Koffi (1990) and as adapted by Karunasagar et al (1996). 200 ml of 0.2 % sterilized tryptic soya broth was taken in a sterilized beaker and inoculated with 16 hr. grown culture of *E.coli*. The plastic coupons (5x5 cm^2) sterilized by 95 % alcohol were dipped into the medium in beaker and incubated at 37°C for 48 hrs. After the incubation the coupons were taken out aseptically and washed with sterilized 0.01 M phosphate buffer pH 6.6 to remove the unattached cells. The coupons were re-immersed in fresh medium with culture, incubated further and washed as before, this cycle of procedure was carried out five times in all.

Test of destruction of *E.coli* cells in biofilm

The coupons with the developed biofilm were dipped in the fermented fluid for different periods of time up to 60 mins. The coupons were then swabbed with sterile cotton swabs, which were transferred to 100 ml physiological saline, shaken vigorously and plated out for enumeration.

Sensory evaluation test

The *M calabura* wine was subjected to sensory evaluation by statistical hedonic rating test to assess consumer acceptability(Ranganna, S.(2005). This was done by its comparison with a popular market sample of white wine (traditional grape wine). The results were analyzed for preference based on wine flavor parameters and overall acceptability with data from 20 untrained panelists, who scored each of the quality parameter of appearance, smell and taste on the scale of 1 to 9 (9 for like extremely and 1 for dislike extremely) . Overall preference was scored as rank 1 and 2 (1 for preference over 2).

Results and Discussion

The mashed fruits of *Muntingia calabura* on analysis was found to contain 8.28 % of total reducing sugar, 0.11 % acidity (as citric acid) and pH 6.21. By supplementation of added cane sugar, the fermentable sugar content was raised to 20.92 %, which was considered desirable to yield an end product wine with around 9-10 % alcohol generally found in traditional wines. In fact, after allowing for 28 days of fermentation a yield of 10 % alcohol (v/v) in the wine was obtained. Data in the Table 1 indicated that in 7 days time alone almost all the sugar had been consumed in the fermentation. On conclusion of the experimental period of 28 days a dry wine was obtained. Judging from low availability of sugar on the 7 th day viz 0.009 %, alcohol increases beyond this period would seem practically nil. The total acid content in the fermented fluid was estimated as 0.31% (data not shown), which is a 3 fold increase over the unfermented material. This is reflected in lowering of pH from 5.63 to 3.80 in the fermented fluid. (Table 1).

The data on phenolics estimated as gallic acid equivalent during the fermentation period is recorded in Fig.1.The concentration of phenolics has shown an increase from 0.82 mg/ml at the start of the fermentation to 1.68 mg/ml on the 28th day, which was more than a 2 fold increase. The data suggested that rapid solubilization of phenolics occurred concomitantly with increase in alcohol concentration and further increases in phenolics content which occurred were during the period when fermentable sugar was practically non-available for formation of alcohol.

A similar trend in the increases in the reducing power measured as tannic acid equivalent was seen (Fig.2). At the start of the fermentation this was 0.8 mg/ml, which by 21st day had increased to 1.3 mg/ml, a 1.625 fold increase. It remained same on 28th day.

DNA damage is considered as the primary event towards mutation and carcinogenic transformation (Halliwell and Gutteridge,1990,1999). The damage to DNA is conventionally assessed by structural or chemical measurements. It is done qualitatively by polyacrylamide gel electrophoresis (Marappan and Srinivas ,2007) or measurement of 8-hydroxy-2'-deoxy guanosine as a product of oxidative damage of DNA (Wei et al 1996). In the present work spectrophotometeric measurement of DNA wherein the reduction in absorbance at 260 nm was taken as indicative of damage and the magnitude of such reduction related to the measure of damage. In this procedure damage to DNA was chemically induced by a ferrous sulfate–ascorbic acid system, where ferrous ion is a known mediator of a reactive oxygen species (ROS) generation (Halliwell and Auroma 1993). Since this reaction is accompanied by ferrous-ferric transition, ascorbic acid was included to reverse it to maintain constant supply of ferrous ion.

The ability of the *Muntingia Calabura* fruit to protect DNA against experimentally induced damage at the start of fermentation was 20.73 % and at the end of experimental period of 28 days the protection seemed total (100 %) (Table 2). It is reasonable to assume that

one or more number of constituents of the fermented fluid were involved in neutralizing the destructive free radical generated by the ferrous–ascorbate system. There have been a few earlier reports of such DNA protection, for example the protective activity of genistein, a soy bean isoflavone (Wei et al, 1996), by extracts of *Stevia rebaudiana* leaves used as a natural artificial sweetener (Ghanta et al,2007) and by *Rosa canina*, a medicinal plant (Kilicgun and Dehen,2009).What may be considered significant in the present work is the enhancement of DNA protective action as a result of alcoholic fermentation of *M.calabura*, which could be both a value addition and of physiological advantage in the wine.

The inhibition of the pathogens, *E.coli, S.typhi* and *Staph. aureus* by the fermented fluid was clearly noticeable in nutrient broth-culture and disc-diffusion test (Table 3). The wine was concentrated to one tenth the volume over the water bath to perform this test. This would have caused all the alcohol to be lost by evaporation. Hence the inhibitory factors were seemingly components inherent to *M. Calabura* fruit, which were solubilized during the fermentation process.

The observation of bactericidal effect of the fermented fluid on *E.coli* in its biofilm form should be regarded more significant, as most pathogenic manifestation are considered to be in biofilm morphology (Fig 3). Two controls were run in the biofilm study. One control was just phosphate-citrate buffer , pH 3.8 (same pH as wine) at 10mM salt content. In this control inactivation of *E.coli* was 8.7% after 60 min presence in the buffer. This may be attributed to the effect of low pH .The second control had in the same buffer an added 10% ethanol (the same amount as in the wine). In this control *E.coli* destruction was found to be much higher , a 68% in 60 min exposure. Indeed, in the test sample with wine, *E.coli* destruction was a substantial, 84% in 60 min exposure. The data showed a distinctive destructive role of *M.calabura* wine on *E.coli* in biofilm morphology.

Ethanol at 10% concentration is not regarded as germicidal in terms of a general purpose disinfectant against a wide spectrum of microorganisms. It is usually 60-85% (v/v) ethanol used in germicidal formulations. It would seem however, that some organisms, such as *E.coli* may be more sensitive to even lower concentration of ethanol as found in the present work.

The results of the hedonic rating test for *M. calabura* wine in comparison with a market sample wine are presented in Table 4 . The data indicated higher rating for smell and taste, but lower rating for appearance of *M. calabura* wine when compared to the market sample. In the preference ranking, *M. calabura* wine scored marginally lower, which was possibly biased by its pale brown color while the market sample was colorless. The check on the fidelity of higher score of *M. calabura* wine in smell and taste attributes was supported by the fiducial limits analysis of the data obtained for the market sample where in the mean score of *M. calabura* wine were found to be well within the range, thus indicating the comparability of *M. calabura* wine with the market sample for acceptability.

The preliminary data of the present study indicated that in wine fermentation, besides alcohol, there are other factors of value which emerge, the fruit itself seemingly being their main source. The beneficial effects of these other factors have been indicated by several workers, who have examined them in isolation, mostly in *in vitro* studies. Whether such factors are available in full measure directly from the fruit needs to be assessed. The present data indicated that they can be released (seen as enhanced) in alcoholic fermentation of *M. calabura* fruit. It is possible that they exist bound or integral to complex macromolecules (eg. polyphenols) and have to be released by enzymatic degradation or conversion. It is known that fruits possess hydrolytic enzyme which can get activated in the mashing process. It would also be of interest to study the role of yeast enzymes as well as of alcohol solutions, as the later may possess ability to release molecules with hydrophobic domains.

The *M. calabura* is a fast growing, draught tolerant plant, capable of thriving in poor soils and it is also acidic and alkaline soils. It is known to be high fruit-yielding, nearly all through the year. In developing countries like India it is being introduced as a shade-provider or as avenue tree and not so much as a valuable fruit-yielder (Chin, 1989; Corner,1997; Morten,1987; The wealth of India,1962)The present report has indicated the potential of *M.calabura* fruit for economic exploitation and development of a possible health–promoting beverage.

Acknowledgement

The authors thank Mr. A.R. Subramanya, CEO, Ganesh Consultancy and Analytical Services (GC&AS) for providing resource and facilities for carrying out this work. Thanks are also due to Mr. B.S. Subhash, Technical Manager, GC&AS for helpful discussions. The kind gift of a transfer of *Staph aureus* MRSA by Dr. Anjali Arun, Chief of laboratory services, Vikram Hospital and Heart care, Mysore, India is gratefully acknowledged.

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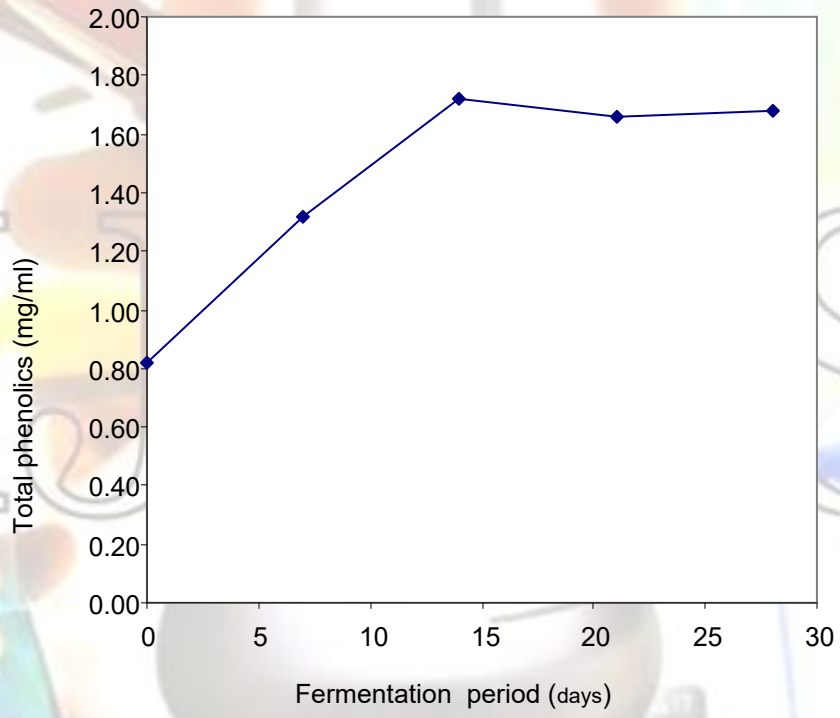
Fig. 1: Total phenolics content of *M.calabura* wine as function of fermentation period



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ISSN: 0976-7126

Fig. 2: Total reducing power of *M.calabura* wine as a function of fermentation period

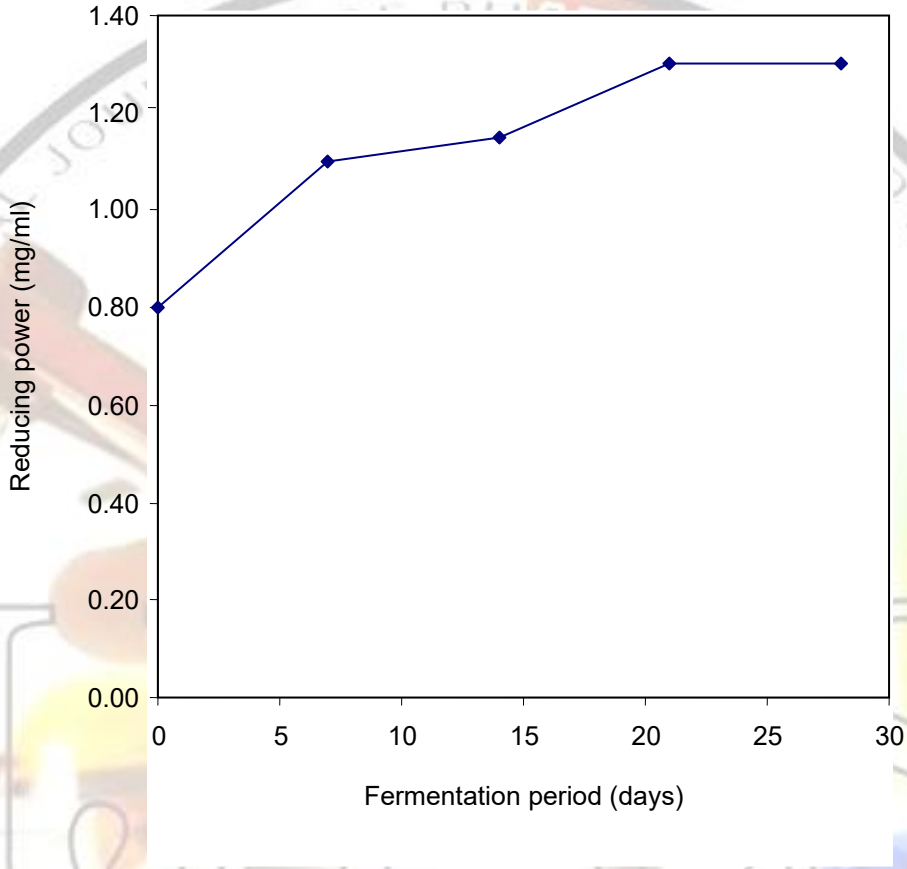

Fig. 3: Biofilm destruction by *M.Calabura* wine

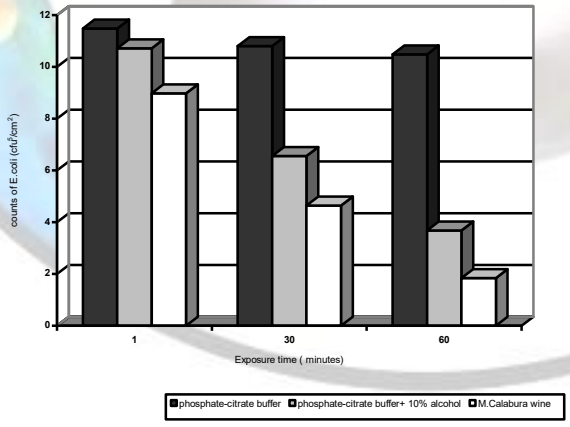

Table 1: The progression of alcoholic fermentation in *M.calabura* wine

Fermentation period (days)	pH	Total reducing sugar (%)	Alcohol % (V/V)
0	5.63	20.920	< 1.0
7	3.65	0.009	NA
14	3.86	0	NA
21	3.89	0	NA
28	3.80	0	10.0

NA: Not analyzed.

Table 2: Protection by *M. calabura* to DNA against chemically induced damage

Treatment [a]	A 260 nm	DNA	
		Destruction %	Protection %
1	0.180	-	-
2	0.151	16.11	0
3	0.157	12.77	20.73
4	0.190	0	100

[a]
1. DNA
2. DNA + (Ferrous sulfate/Ascorbic acid)
3. DNA + (Ferrous sulfate/Ascorbic acid) + (*M. calabura* mash prior to fermentation)
4. DNA + (Ferrous sulfate/Ascorbic acid) + (*M. calabura* mash after fermentation)

Table 3: Influence of *M.calabura* wine concentration on inhibition of bacterial cultures in nutrient broth

Sample Volume (µl)	Growth		
	E.coli	*Staph aureus*	*Salmonella typhi*
0 (control)	+	+	+
100	+	+	+
200	+	+	+
300	_	_	+
400	_	_	_
500	_	_	_

+ Growth , - No growth.

Table 4: Analysis of hedonic rating of *M.calabura* wine in comparison to a market sample grape white wine

Evaluation Item	Score		Fiducial limit range for market sample wine	
	Market sample	*M.calabura* wine	at 5 % level	at 1 % level
Appearance (mean score)	6.75	4.50	5.98-7.52	5.81- 7.69
Smell (mean score)	5.65	6.20	4.92 – 6.38	4.76 – 6.54
Taste (mean score)	5.55	6.35	4.77 – 6.33	4.61 – 6.49
Rank (∑ x)	27	29	----	----