Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 2772-2777
Received: 16-03-2019
Accepted: 18-04-2019

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Preparation and evaluation of wine from *Rhododenron arboreum* and *Madhuca longifolia* flowers juice

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Abstract
Rhododendron arboreum and *Madhuca longifolia* are traditionally medical plants used in the production of wine in our study. Six different sets of samples were prepared containing different concentration of both the flowers juice. Parameters studied included Total sugar content, alcohol content, titratable acidity, sensory analysis, Total phenol content, antioxidant activity and antimicrobial activity. The results revealed that sugar content decreases and there is increase in alcohol content as well as acidity percentage with increase in fermentation period. It has been seen that with increase in mahua flowers content alcohol content increases. We have also seen that there is linear increase in alcohol content up to Day 8 of fermentation period and after that up to Day 10 there is little or no increase in alcohol content was seen and after that up to Day 14 very little amount of increase in alcohol content was seen. Phenol content (13.40-14.45%) decreases with fermentation in all the samples. Almost all the samples show good antioxidant and antimicrobial activity. The study concluded that yeast is highly efficient in utilizing flowers juice to produce wine with substantial alcohol content, markedly good properties and was highly acceptable. Based upon the sensory attributes RMW 3 is the most acceptable sample with good physical appearance, taste and aroma.

Keywords: Wine, *Rhododenron arboretum, Madhuca longifolia* flowers juice

Introduction
Wine is an alcoholic beverage which is prepared from a variety of fruit juices by the fermentative action of selected yeast. Traditionally apple, pear, grape and berry were preferred in making wine and mainly available in Europe, Middle East, America or North and South Africa (Kunkee *et al*., 1994) [1]. Beside all these fruits, other flavours can also be incorporate by using various types of fruits, leaves or plants having number of medicinal properties. In ancient times alcoholic beverages are also being used for their medicinal values, so use of flowers named *Madhuca longifolia* (Mahua) and *Rhododendron arboreum* in wine preparation process not only provides good amount of alcohol production but also induces health benefits of these flowers with variation of ratios of juice. *Rhododendron arboreum* flowers are a rich source of carbohydrates, amino acids, polyphenols, flavones, coumaric acid, ursolic acid and resins (Cho *et al*., 2008; Kashyap *et al*., 2016) [2, 3]. Epicatechin, syringic acid, quercetin-3-*O*-galactoside and quercitrin has been determined from the leaves of *Rhododendron arboreum*, *Rhododendron campanulatum* and *Rhododendron anthopogon* (Sharma *et al*., 2010) [4]. Flowers of *Rhododendron arboreum* have antioxidant (kashyap et. al., 2017) [5] anti-inflammatory and cholinergic activity (Agrawal *et al*., (1988); Tripathi *et al*., (1992) [6, 7] and used for checking diarrhoea and blood dysentery (Bhattacharjee *et al*., 1998) [8]. *Madhuca longifolia* (Mahua) belongs to family Sapotaceae and commonly known as the Butter nut tree. Mahua flowers are rich source of sugars and used in production of vinegar (Wealth of India, 1964) [9], and thus used in alcohol production at low cost.Fermentation is very simple and cost effective technique which if done avoids the other preservation techniques and makes the product shelf life better. In addition of providing health benefits to consumers, use of *Rhododendron arboreum* and *Madhuca longifolia* flowers for the production of wine will also create employment, income generation for farmers.

Materials and methods
Procurement of flowers
Flowers of *Rhododendron arboreum* were collected from Mandi district, Himachal Pradesh and *Madhuca longifolia* flowers were taken from Bhagalpur district, Bihar.

Preparation of flowers juice

Freshly picked flowers were used and investigated for dirt and blemishes and juice was extracted from damage free flowers by boiling them in water in the ratio of 1:2. After boiling, extract was filter by using muslin cloth and filled in clean dry bottle and kept at 2-4 °C till further use (Fig 1)

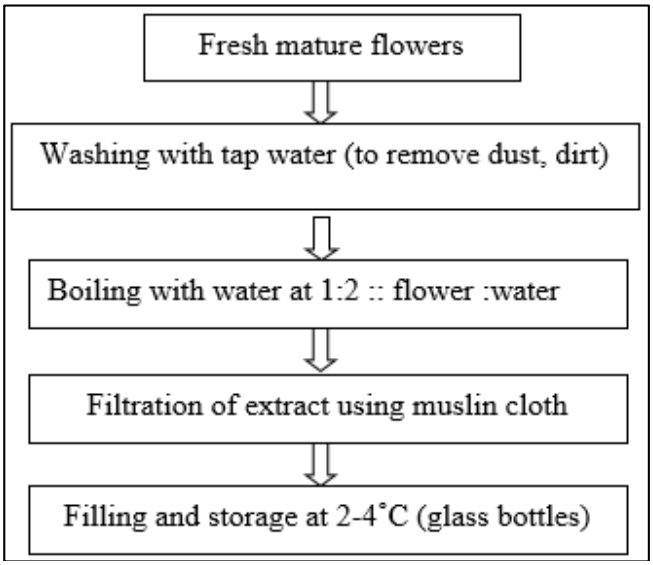

Fig 1: Preparation of juice from flowers

Formulations of *rhododendron arboreum* and *Madhuca longifolia* flowers juice

Six different samples were prepared by blending different proportion of juice from both flowers (Table 1).

Table1: Blended samples of *Rhododendron arboreum* and *Madhuca longifolia* flowers juice

Sample	*Rhododendron arboreum* flowers juice (ml)	*Madhuca longifolia* flowers juice(ml)
RMW1	90	10
RMW2	80	20
RMW3	70	30
RMW4	60	40
RMW5	50	50
RMW6	40	60

RMW- Rhododendron Mahua wine

Analysis

Blended juice was analyzed for various physico-chemical characteristics viz., total sugars and titratable acidity. Wine prepared during study was analysed for various physico-chemical and sensory characteristics viz., Ethanol, Titrable acidity, colour, total sugar, total phenols, antioxidant activity and antimicrobial activity.

Determination of alcoholic content

One millilitre of the fermented wash was taken in 500ml Pyrex distillation flask containing 30 ml of distilled water. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulphuric acid and volume raised to 1 litre). About 20 ml of distillate was collected in each sample and the flasks were kept in a water bath maintained at 62.5 ºC for 20 minutes. The flasks were cooled to room temperature and the volume raised to 50 ml. 5 ml of this solution was diluted with 5ml of distilled water for measuring the optical density at 600nm using a spectrophotometer(Caputi *et al*., 1968) [10].

Total sugar measurement

Phenol sulphuric acid method is widely used for the determination of carbohydrates content in solution (DuBois *et al*., 1956) [11]. A stock solution of sample was prepared by dissolving 0.1ml of sample in 1 L of water. A 2 mL aliquot from stock solution is mixed with 1 mL of 5% aqueous solution of phenol in a test tube. Subsequently, 5 mL of concentrated sulphuric acid is added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they are vortexed for 30s and placed for 20 min in a water bath at room temperature for colour development. Then, absorbance was measured at 490 nm. Reference solutions are prepared in identical manner as above, except that the 2 mL aliquot of carbohydrate is replaced by distilled water. Glucose is used as standard. The concentrations that were prepared for this study are: 0.01, 0.03, 0.05, 0.07 and 0.09g/L, Titrable acidity

Titrable acidity was estimated by titrating a known aliquot of the sample against N/10 NaOH solution using phenolphthalein as an indicator. The total titratable acidity was calculated and expressed as per cent citric acid (A.O.A.C., 1980) [12].

Test for phenol

Total phenolic content was determined according to Folin–Ciocalteu's reagent method (McDonald *et al*., 2001) [13]. 100µl of wine extract was mixed with 250µl of Folin-Ciocalteu reagent. After 5 minutes, add 1.5 ml 20% Na_2CO_3 solution and makeup volume up to 5ml. Now, allowed it to stand for 2 hours and absorbance of the reaction mixture was measured at 760 nm. Gallic acid was taken as reference standard. Amount of total phenol was measured in milligram Gallic acid equivalent (mg GAE)/g of dried flowers extract.

Antioxidant activity

The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams *et al* (1995) [14]. Stock solution of DPPH (24 mg DPPH with 100mL methanol) was prepared and kept at -20 °C until needed. Working solution was prepared by mixing 10ml stock solution with 45ml ethanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. The reaction mixture consists of 150 µl wine extract and 2850 mL DPPH solution and allowed to stand for 24 h in the dark. Absorbance was measured at 515nm. Trolox was taken as standard for preparation of standard curve and results were expressed in mMTrolox equivalents/g fresh mass.

Determination of antimicrobial activity

Test microorganism

The antimicrobial activity of flower extract was assessed against six bacteria species: *Bacillus subtilus, Bacillus cereus, Staphylococcus aureus* (Gram positive) and*Escherichia coli, Salmonella enterica, Shigellaflexineri (Gram negative).* Cultures were incubated overnight at 37 °C.

Antimicrobial screening

Antibacterial activity assay was carried out using agar well diffusion assay (Guitrezz, 2008) [15]. 100 µl of bacterial cell suspension was spread over the nutrient agar plate and 6 mm wells were punched into the agar with a sterile cock borer. 50 µl of wine extract of different concentration were added to each well. Agar plates were now incubated at 37 °C overnight. Triplicate plates of each organism were prepared. 10µg/ml of gentamicin was used as positive controls of inhibition.

Results and Discussion

Alcoholic content

The increase in alcohol content of wine samples is shown in Fig 2. From day 1 to day 8, there was gradual increase in alcohol content of the wine samples and linear increase from day 8 to day 14 of the fermentation period. Maximum alcohol content (6.3%) is found in sample RMW 6 which 60% *Madhuca longifolia* juice and 40% *Rhododendron arboreum* juice. From the figure 4, we can conclude that with increase in percentage of *Madhuca longifolia* juice alcohol percentage increase. In general, alcohol content gradually increases as the fermentation proceeds. This is due to utilization of sugar by yeast in favourable must conditions which includes temperature of must, adequate amount of sugar, specific acid and pH conditions and absence of wild yeast and other microorganisms. The internal temperature of the must is very critical during fermentation. This increase in alcohol content may due to absence of wild microbes like lactobacilli because these unwanted microbes causes homo-lactic fermentation and may also be utilized by other fungal species to produce acids (Dikinson *et al*., 1999; Axelsson *et al*., 1998) [16, 17]. At the end of fermentation period with the rise in alcohol content and decrease in pH causes linear increase in alcohol content due to slow action of yeast (Mountney *et al*., 1988) [18].

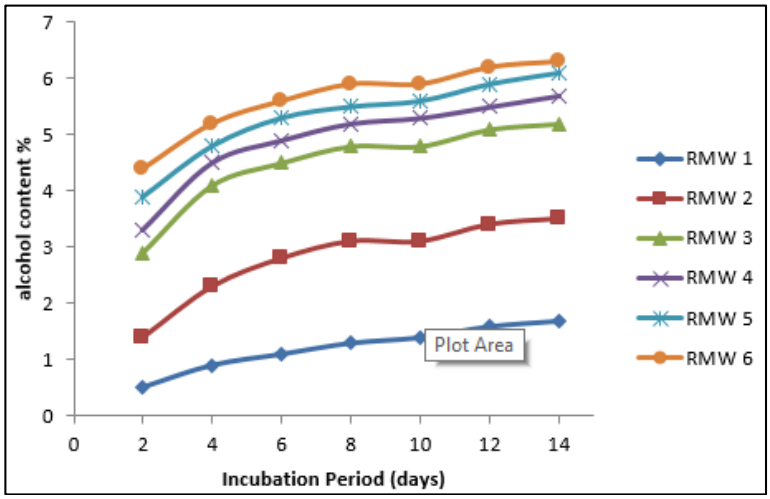

Fig 2: Change in alcohol content in different wine samples

Sugar content:

Sugar content gradually decreases from day 1 to day 14 of the fermentation period (Fig 3). The total sugar content of the must decreased from 41.63 mg/ml to 6.05mg/ml in RMW 6, 38.94 mg/ml to 4.22 mg/ml in RMW 5, 36.37 mg/ml to 3.01 mg/ml in RMW 4, 33.03 mg/ml to 2.35 mg/ml in RMW 3, 28.42 mg/ml to 1.74 mg/ml in RMW 2 and 25.86 mg/ml to 1.19 mg/ml in RME 1. Total sugar showed a gradual reduction during the fermentation period because the sugar gets converted into alcohol and carbon dioxide.As the fermentation period proceed consumption of sugar increases (Rooyen *et al*., 1982; Sulz *et al*., 2011) [19, 20]. Steinkraus (1992) [21] reported that sucrose level decreases from 15% to 1% during the fermentation period while working on wine production from *Agave Americana.*

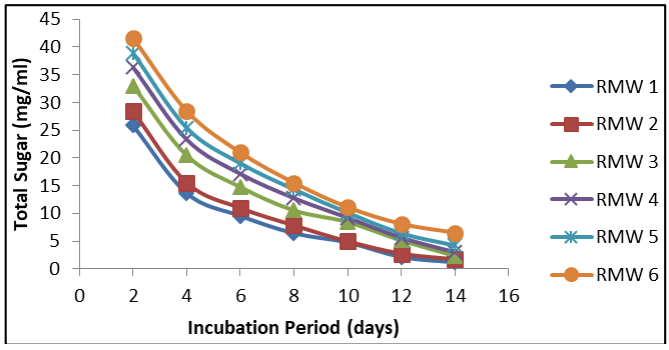

Fig 3: Change in sugar content in different wine samples

Titrable acidity

The change in titratable acidity with fermentation period of different samples is shown in Fig 4. Total titratable acidity gradually increases from day 1 to day 14 of the fermentation period. In all the different must samples total titratable acidity percentage ranges from 0.25% to 0.54%. In the samples RMW 3 to RMW 6 there is very less difference in final acidity percentage i.e. RMW3 (0.52%), RMW4 (0.52%), RMW5 (0.53%), RMW6 (0.54%). Wine quality is directly affected by acid and with increase in titratable acidity quality of wine also increases. Citric acid, tartaric acid and some amount of lactic acid is generally present in wine in which lactic acid sometimes undergoes malo-lactic fermentation by replacing malic acid (Fleet *et al*., 1998) [22]. The presence of acids in wine enhances the taste, aroma and preservative properties of the wine (Wimalsiri *et al*, 1971) [23].

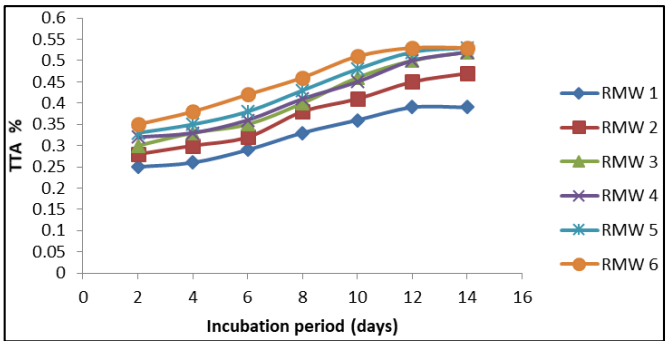

Fig 4: Change in Titrable acidity in different wine samples

Total phenol content

The total phenolic content of different must samples is shown in Table 2. After fermentation, there was depletion in total phenolic content in all the fermented samples. In all the samples the phenolic content depleted by 13.40% to 14.45% with maximum depletion seen in RMW6 and minimum in RMW1. It has been seen that with increase in *Madhuca*

longifolia juice total phenolic content decreases. It may be due to greater fermentation efficiency due to larger sugar amount present and greater percentage formation of alcohol content. In Table 2 we can see that in sample RMW1 to RMW 4 there is not much decrease in alcohol percentage. The observed variation in phenolic content after the fermentation may be correlated with several mechanisms- including adsorption of polyphenols onto yeast cell walls, condensation and polymerisation reactions and enzymatic activity (Czyzowska and Pogorzelski, 2004; Ginjom*et al*, 2011; Perez *et al*, 2011) [24, 26].Phenolic components greatly contribute to the organoleptic characteristics of fermented products like wine such as color, astringency and aroma (Lopez-Velez *et al*, 2003) [27]. And have also been associated with beneficial physiological effects like protective effects against oxidative stress and hyper cholesterolaemia. (Negi *et al*, 2013) [28].

Table 2: Reduction in Total Phenol content after fermentation

Samples	RMW1	RMW2	RMW3	RMW4	RMW5	RMW6
TPC (mg GAE/L)	807.8	805.1	803.5	801.2	797.4	796.1
Post fermentation Decrease (%)	13.40	13.43	13.69	13.76	14.13	14.45

Antioxidant activity

No significant difference was observed in all the samples of wine. RMW 1 is found to have highest antioxidant activity (6.98 mmol TE/L) and RMW 6 is having lowest (5.63 mmol TE/L) Antioxidant activity.

This assay is based on ability of reducing the violet colour as sign of antioxidant activity. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is the stable free radical that contain a decentralized electron. When DPPH is mixed with wine extract it gives hydrogen atom to reduce the free radical and changes colour from deep violet to yellow. Greater change in colour increase good antioxidant activity (Naik *et al*., 1993) [29]. The scavenging potential of wine extracts was calculated in trolox equivalents as total antioxidant potential along with percent inhibition capacity of extracts (Table 3). In comparison with the control (trolox), the increase in the antiradical activity against the DPPH depends on the concentration of the different extracts.

Table 3: Antioxidant activity of different wine samples

Sample	Antioxidant activity(mmol TE/L)
RMW 1	6.98
RMW 2	6.74
RMW 3	6.59
RMW 4	6.14
RMW 5	5.86
RMW 6	5.63

Antimicrobial activity

All the samples of wine showed antimicrobial activity. The antibacterial activity of different wine samples was determined using Well Diffusion assay. The results of testing of the wine samples extracts for antimicrobial activities against bacterial species are summarized in Table 4. All Gram positive bacteria and Gram negative bacteria tested were susceptible to different wine samples. As all the different samples are rich in phenolic compounds, hence showing high antimicrobial properties of the sample extracts. The main antimicrobial mechanism of phenolics is due to presence of citoplasmatic membrane and the outer lipidic membrane of the Gram negative bacteria which provides additional protection to these bacteria (Axelsson *et al*., 1998) [17]. The best antibacterial activity was shown by RMW 5, RMW6. There is not much difference in antimicrobial activity of samples RMW3 to RMW 6. *Bacillus subtilis* and *staphylococcus aureus* exhibited the highest sensitivity to all the wine extracts with maximum zone of inhibition (21mm) in case of RMW 6. All wine extracts exhibited some kind of antibacterial activity against both Gram-positive and Gram-negative strains. In all extracts, gram positive bacteria shows higher zone of inhibition as compared to Gram-negative bacteria which indicates the greater sensitivity of Gram-positive bacteria than gram negative bacteria. It is due to different structure of cell wall of both bacteria. In case of Gram-negative bacteria they have more complex structure with presence of outer lipid membrane which is less permeable as compared to less complex Gram-positive bacteria membrane which is more permeable to antimicrobial compounds.

Table 4: Antimicrobial activity of different wine samples

S. No.	Sample	Diameter of inhibition zone (mm)					
		B. subtilus	***B. cereus***	***S. aureus***	***E. coli***	***S. enterica***	***S. flexineri***
1	RMW 1	17	15	16	12	10	12
2	RMW 2	18	15	17	12	12	13
3	RMW 3	19	17	19	13	14	15
4	RMW 4	20	17	19	14	14	16
5	RMW 5	21	18	20	14	15	16
6	RMW 6	21	20	21	15	16	17

Sensory analysis

Sensory evaluation of the wine from different samples is presented in Fig 5. Is has been seen in the tables all the wine samples had a score between 10 to 15 which implies all samples were fall in the commercial and standard wine category. Comparison is done with commercial wine sold in the market which is scored 16. From the table, we concluded that sample RMW 3 is the best wine sample got the score of 15 and liked by evaluators. Due to its appearance, flavour and aroma it had been evaluated as best wine sample. Acceptance of wine depends on its flavour, aroma and appearance. With ageing and racking of wine quality of wine is enhanced. During ageing and maturation of wine whether occurs in bottles or barrels many chemical reactions occurs like oxidation which enhances taste, aroma and preservative

properties of wine by forming various esters and aldehydes Wimalsiri *et al.*, (1971) [23] and Au Du, (2010) [30].

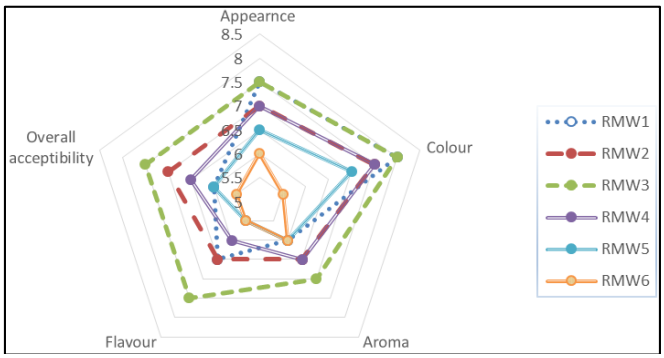

Fig 5: Sensory analysis of different wine samples

Conclusion

The seasonality of *Rhododendron arboreum* and *Madhuca longifolia* flowers and absence of industrial utilization makes these medically important flowers underutilized. The study includes the utilization of these flowers in making wine. The wine produced has low alcohol content, good aroma and colour. Due to which it is highly acceptable. Based on sensory parameters and chemical analysis RMW 3 sample of wine which contains 30% mahua flowers juice and 70% Rhododendron flowers juice is the best combination among all the six samples prepared. Wine shows good amount of phenolic content which reveals that it has good antioxidant activity and antimicrobial activity. The high antioxidant effect has an add-on advantage in promoting health of an individual. The abundant availability of both flowers during season as raw material is helpful in reducing the time, income, costashipping charges to be used in production of wine at industrial level.

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