Effect of Total Flavonois and Methanol Extract of *Phyllanthus mellerianus* leaves on the Fermentation of *Elaeis Guineensis* Sap

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ABSTRACT---- ***The rate studies of palm wine from Elaeis guineensis is of major importance in determining the extent of inhibition and activation of microbes in Elaeis guineensis sap fermentation. Saps from E. guineensis were dosed with 10-80mg of 'total flavonoids' and methanol extract of Phyllanthus mellerianus leaves. The rates of their fermentation were monitored kinetically. Results showed that the 60mg dose of 'total flavonoids 'increased the rate of fermentation significantly. Fermentation was also increased with 40mg of methanol extract dose, but not as much as with '40-60mg/100mL sap than that produced during the fermentation of the pure sap (control). However, methanol as fermentation product was absent, so the sap and the dosed saps are not methanol toxic. While iso-propanol as one of the fermentation products, was present in both the pure and dosed wine.***

Keywords---- *Phyllanthus mellerianus*, 'total flavonoids', methanol extract, *Elaeis guineensis*, sap, fermentation

1. INTRODUCTION

Palm wine is an alcoholic beverage produced by fermentation of sap of various palms such as *Elaeis guineensis, Raphia hookeri*, etc by natural microbes (Obire, 2005). The unfermented sap is clean, sugary taste, colourless syrup containing about 10-12% sugar, which is mainly sucrose (Bassir, 1962; Okafor, 1975a), minimal invert sugar (Less than 0.5%) small amounts of proteins, gums and mineral (Opara et al., 2013). For palm wine to be acceptable, it must not only be whitish in appearance due to the increased microbial suspension resulting from the prolific growth of the fermenting organism (Ezeaga and Tafunso, 2003) but must also have a pleasant sugary taste and exhibit vigorous effervescence (Bassir, 1962, Okafor, 1965a, Faparusi, 1973).

Palm wine is consumed in various parts of the tropical world including South America, Asia and Africa (Chandrasekhar et al., 2012). Palm wine differs from conventional beers, table wines and burukutu produced in the modern and native brewery and winery in different ways.

The media for which beers and burukutu are usually grains, for wines, grapes and fruit juice are used, while honey is fermented directly. The basic principle is however the same: a sugar solution is fermented, essentially by yeast and several bacterial flora (Nester et al., 2004; Orimaiye, 1997; Ejiofor et al., 1994).

Generally the brands of palm wine consumed in this part of the world have several nutritive, medicinal, religious, social and industrial uses which have been reported elsewhere (Faparunsi 1966; Odeyeni, 1977; Iheonu 2000; Ikenebomeh and Omayli, 1988; Uzogara et al., 1990), to have increasingly enhanced the demand for this natural product. Although attempts have been made towards the inhibition of the microbial activities and extend the shelf-life of the palm wine through bottling, use of chemical additives and addition of plant extracts have greatly affected the organoleptic quality and quantity of the product (Orimaiye, 19997; Iheoun, 2000; Nwokeke, 2001; Obire, 2005; Chime et al., 2007).

Several flavonoids and phytoconstituents from edible and medicinal plants have been reported to posses potent anti-microbial activities (Mishra et al., 2009; Mishra et al., 2013; Tsuchiya and Linuma, 2000; Meda et al., 2013; Cushnie and Lamb, 2005). Therefore this study was primarily carried out to assess the effect of various concentrations of 'total flavonoids' and methanol extract of *Phyllanthus mellerianus* leaves, on 24hrs fermented palm wine (*Elaeis guineensis*).

2. MATERIALS AND METHOD

Sample collection

Fresh palm wine sample from oil palm tree (E. guineensis) was brought from a local palm wine tapper in Udi Town of Enugu state, using pre-sterilized labelled sample container (4L) in iced cooler and immediately transported to the laboratory.

Plant extracts preparation

The plant materials were processed into extracts before use but they were first examined visually for the presence of extraneous materials such as dirt, insect larvae or eggs as well as diseased parts and then removed. The sorted plant leaves were washed in running water to remove dust and other dirt. They were allowed to drain dry and then spread in a laboratory tray and sun dried until they were brittle enough to grind. The dried leaves were ground in a laboratory mill to obtain powdered sample.

Successive extraction and phytochemistry

About 48g of the powdered plant leaves were successively extracted with n-hexane, ethyl acetate and methanol. After extraction the extracts were concentrated under reduced pressure using a rotary vacuum evaporator. Phytochemical screening of the crude extracts were carried out using standard methods described elsewhere (Sofowara, 1993; Trease and Evans, 2002; Perinos and Quimbly, 1967) each plant extract was screened for the presence of some compounds including saponin glycoside, steroid/triterpenoids, glycoside, anthracenes, digitals glycoside, tannins, pseudo tannins, flavonoids, resins, alkaloids and volatile oils.

Isolation and total flavonoids determination

The powdered sample (10g) was refluxed with 100mL of 95% ethanol for 1hr at 80^{o}C and allowed to cool down to room temperature. The cold extract was filtered with a Watmann filter paper No.1. The isolate was evaporated to dryness under reduced pressure using a rotary evaporator.

Standard curve was prepared by adding 0, 0.1, 0.2, 0.3, 0.4 and 0.5mL of 1mg/mL Rutin to six test tubes numbered 0-5 respectively. To test tubes 6 and 7, were added 1mL of each of the sample isolate (1mg/mL). To these test tubes were added distilled water to make the volume up to 1mL. Then 0.5mL of $NaNO_2$ was also added to each test-tube and shaken for 5min. Four milliliter of 4% NaOH was also added and shaken for 15 mins. Test tube 0 was used to zero the UV/visible spectrophotometer and the absorbance determined at 510nm (li et al., 2003).

Fermentation Studies of Palm Wine

The experimental procedure of Agie et al; 2000 was used. Exactly 100mL of E. guineenus sap were dosed with 0.0, 10, 20, 40, 60 and 80 mg of 'total flavonoids' and 10, 20, 30, 40 and 50 mg of methanol extract of *P. mellerianus* leaves. The rate of fermentation was determined by monitoring the volume of carbon dioxide evolved during fermentation and reading taken every 1hr.

Total Reducing Sugar Determination

The Fehling's solution method as described by Anon, 1970 was adopted for total reducing sugar content of the sap and 24hr fermented palm wine respectively. Twenty five milliliter of mixed Fehling's solutions A and B (50/50 v/v) was pipetted into a 25mL of water in a conical flask and boiled. The boiled mixture was titrated with standard glucose solution (0.2g/250ml) using methylene blue as indicator till a brick colour was obtained. The process was repeated with fresh sap and 24hr fermented palm wine respectively.

Determination of Percentage Alcohol

Fifty milliliter of distilled water was added to 100mL of the fermented palm wine and those dosed with plant 'total flavonoids' and methanol extract respectively. Then 95mL was distilled into a measuring cylinder and the volume made up to 100mL with distilled water. The percentage alcohol produced during the fermentation process were found using alcoholmeter (Gay Lussac Model N0. 6181)

Test for Methanol as Fermentation product

To 5mL of each distillate was treated with 2mL of reagent A (3g potassium permanganate and 40% Phosphoric acid solution) and allowed to stand for 10minutes. Then 2mL of reagent B (5% Oxalic acid in sulphuric acid solution) was added followed by 5mL of Schiff's reagent and observed for violet colouration for presence of methanol.

Test for Iso-propanol as Fermentation product

Five milliliter of each distillate was added to 5mL of acidified mercuric sulphide (5g mercuric oxide dissolved in 40mL of water and stirred then 20mL of concentrated sulphuric acid was added gradually and the volume made up to 100mL) and heated in boiling water bath for 3 minutes. The appearance of white precipitate is a positive test.

3. RESULT AND DISCUSSIONS

Tables II and III show the result obtained when 0.0, 10, 20, 40, 60 and 80mg of 'total flavonoids' and 10, 20, 30, 40 and 50mg of methanol extract were dosed to Elaeis guineensis sap prior to fermentation. There is no significant effect on the volume of CO_2 produced from the control and 10, 20, 40mg of 'total flavonoids' of *P. mellirianus* leaves but there is a remarkable increase on the volume of CO_2 produced from 60mg and decrease in 80mg from the control.

However, the rate of fermentation in pure Elaeis sap (control) did not vary appreciably from the dosed saps with methanol extract except with 40mg.

Table 1 showed the results of the phytochemical screening of *P. mellirianus* leaves using n-Hexane, ethyl acetate and methanol. N-Hexane extract shows the presence of digitalis glycosides only. Ethyl acetate extract gave glycosides (general) and volatile oils while the methanol extract gave saponin (general), saponin glycoside, glycoside (general), anthracene, tannins and flavonoids.

Tables 2 and 3 show the results obtained when *E. guineenus* sap was dosed with 0.0,10,20,40,60 and 80 mg of total flavonoids and 10,20,30,40, and 50 mg of methanol extract prior to fermentation and their fermentation rate constants respectively.

The observed increase in the rate of fermentation of the sap dosed with 60mg of 'total flavanoids' of *P. mellirianus* leaves might be attributed to the increased activities of microbes in the sap (Faparusi and Bassir, 1971; Okafor, 1968). It is likely that 60mg/100mL of *E. guineenus* sap is the optimum concentration of the 'total flavonoids' that activates the microbes and hence speed up the fermentation process. Figs 1 and 2 show the result obtained for the two saps, dosed with various amount of 'total flavonoids' and methanol extract including the control. The number of moles of CO_2 liberated over time during fermentation was plotted against time (Agu et al., 2000). It was clear that the control produced the least amount of CO_2 (Fig. 2). The addition of 10-80mg of 'total flavonoid' of *P. mellirianus* affects the rate profile against that of the control (in which no 'total flavonoids' and methanol extract of *P. mellerianus* leaves were dosed). In the case of those dosed with methanol extract, the rate profile for methanol extract dosed of 10-50mg deviated slightly from those of the control. This observation might be explained from the point of view of the fact that only methanol extract contain saponin, saponin glycoside, anthracene, tannins, and flavonoids. (Table 1).

The fermentation rate constant and the percent alcohol produced during the fermentation processes were recorded (Table 2 and 3). It is clear from the rate constant that the rate of fermentation is slightly enhanced with the addition of the 'total flavonoids' and methanol extract of *P. mellerianus* leaves. However, with respect to the amount of alcohol produced, it was found that 6mg of 'total flavonoids' and 50mg of methanol extract generated more alcohol (5%) followed by 40mg of methanol extract (4%) than the control (3%). It is possible that the 'total flavonoids' and methanol extract of *P. mellerianus* enhances the rate of fermentation and percent alcohol by making the microbes to convert the sugar into alcohol and other products by microbial activities and oxidation (Agu et al., 2000; Okafor, 1974; Illao, 1981; Vanpee and Swing, 1971). The percent alcohol produced during the fermentation of the 60mg 'total flavonoids' and 40mg of methanol extract dosed sap of Elaeis show clearly that for every time between 0.00 – 3.6 x 10^3 Seconds, the percent alcohol increased more rapidly than the pure sap (control) (Fig. 1 and 2).

Table 1. Phytochemical screening of *Phyllanthus mellerianus* leaves

PHYTO CHEMICALS	N-HEXANL EXTRACT	ETHYLACETATE EXTRACT	METHANOL EXTRACT
Saponin (general)	-	-	+
Saponin glycoside	-	-	+
Steroid/triterpenoids	-	-	-
Glycosides (general)	-	+	+
Digitalis glycoside	+	-	-
Anthracene	-	-	+
Tannins	-	-	+
Hydrolysable tannins	-	-	-
Pseudo tannins	-	-	-
Flavonoids	-	-	+
Resins	-	-	-
Alkaloids	-	-	-
Volatile oils	-	+	-

Key: + = positive, - = negative

Table 1 showed the results of the phytochemical screening of *P. mellirianus* leaves using n-Hexane, ethyl acetate and methanol. N-Hexane extract shows the presence of digitalis glycosides only. Ethyl acetate extract gave glycosides (general) and volatile oils while the methanol extract gave saponin (general), saponin glycoside, glycoside (general), anthracene, tannins and flavonoids.

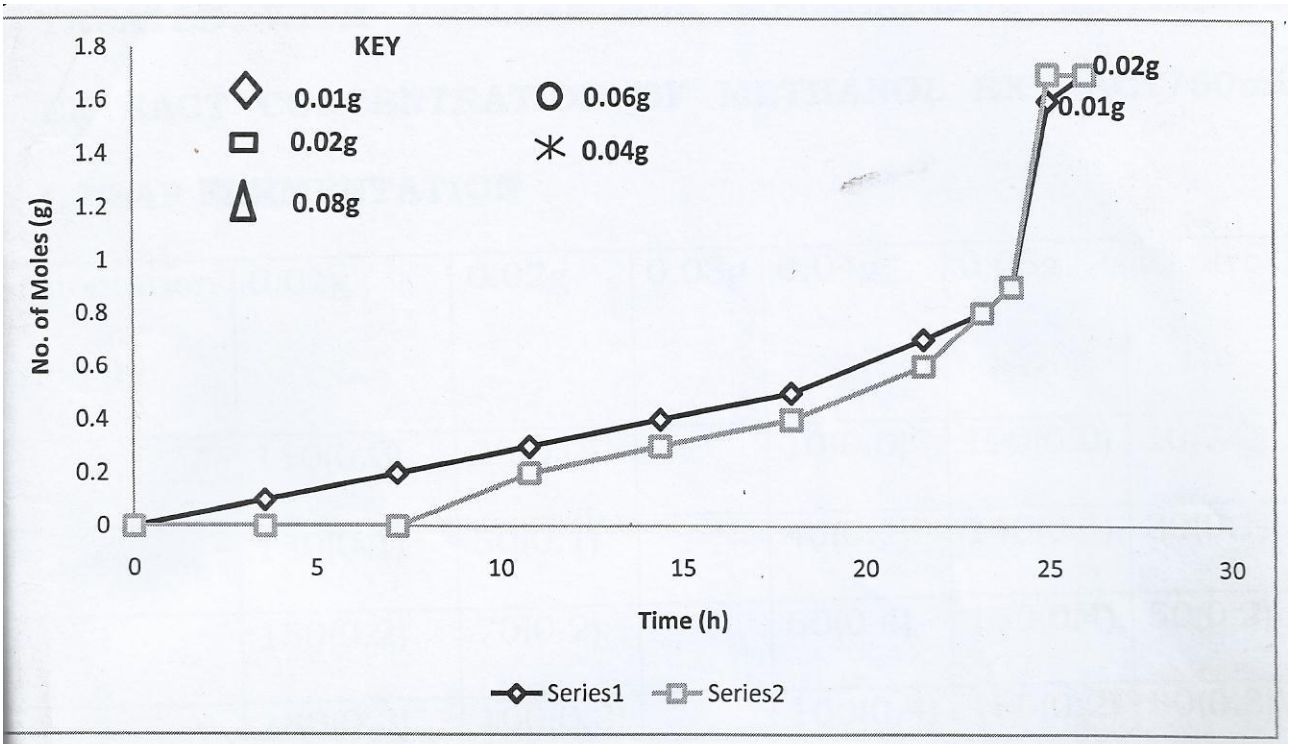
Tables 2 and 3 show the results obtained when *E. guineenus* sap was dosed with 0.0,10,20,40,60 and 80 mg of total flavonoids and 10,20,30,40, and 50 mg of methanol extract prior to fermentation and their fermentation rate constants respectively.

TABLE 2. Volume of $C0_2$ (cm^3) Evolved during *E. guineenus* sap fermentation treated with various concentration (mg) of 'total flavonoids' and methanol extract of Phyllanthus mellerianus leaves/100mL sap

Fermentation period (h)	Control	Total flavonoids (mg)					Methanol extract (mg)				
		10	20	40	60	80	10	20	30	40	50
0	0.0	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	---	0(0.0)	0(0.0)
1	20(0.1)	30(0.1)	30(0.1)	20(0.1)	40(0.2**)**	20(0.0)	20(0.1)	30(0.1)	---	30(0.1)	20(0.1)
2	40(0.2)	40(0.2)	40(0.2)	40(0.2)	60(0.2)	40(0.2)	40(0.2)	50(0.2)	---	50(0.2)	30(0.1)
3	70(0.3)	70(0.3)	40(0.2)	60(0.2)	90(0.4)	70(0.3)	70(0.3)	80(0.3)	---	90(0.4)	60(0.2)
4	100(0.4)	100(0.4)	70(0.3)	90(0.4)	120(0.5)	100(0.4)	110(0.4)	120(0.5)	---	130(0.5)	100(0.4)
5	140(0.6)	130(0.5)	110(0.4)	130(0.5)	170(0.7)	150(0.6)	160(0.6)	160(0.6)	---	170(0.7)	140(0.6)
6	190(0.8)	170(0.7)	150(0.6)	170(0.7)	210(0.9)	200(0.8)	210(0.9)	220(0.9)	---	220(0.9)	190(0.8)
7	230(0.9)	200(0.8)	190(0.8)	220(0.9)	250(1.0)	250(1.0)	260(1.1)	270(1.1)	---	270(1.1)	240(1.0)
8	270(1.1)	230(0.9)	220(0.9)	250(1.0)	300(1.2)	270(1.1)	290(1.2)	310(1.3)	---	330(1.1)	290(1.2)
24	440(1.9)	400(1.6)	420(1.7)	440(1.8)	580(2.3)	240(1.0)	450(1.8)	560(2.3)	---	590(2.4)	520(2.1)
25	440(1.9)	410(1.7)	430(1.7)	450(1.8)	590(2.4)	240(1.0)	450(1.8)	560(2.3)	---	590(2.4)	530(2.1)

Key: Values in parenthesis are moles of CO_2 produced

Other fermentation products studies revealed that Iso-propanol was present in both the control and those dosed with 'total flavonoids' and methanol extract of *P. mellerianus* leaves while methanol as fermentation product was absent in both control and dosed sap. The absence of methanol as fermentation product revealed that palm wine is not toxic to human (Table 4).



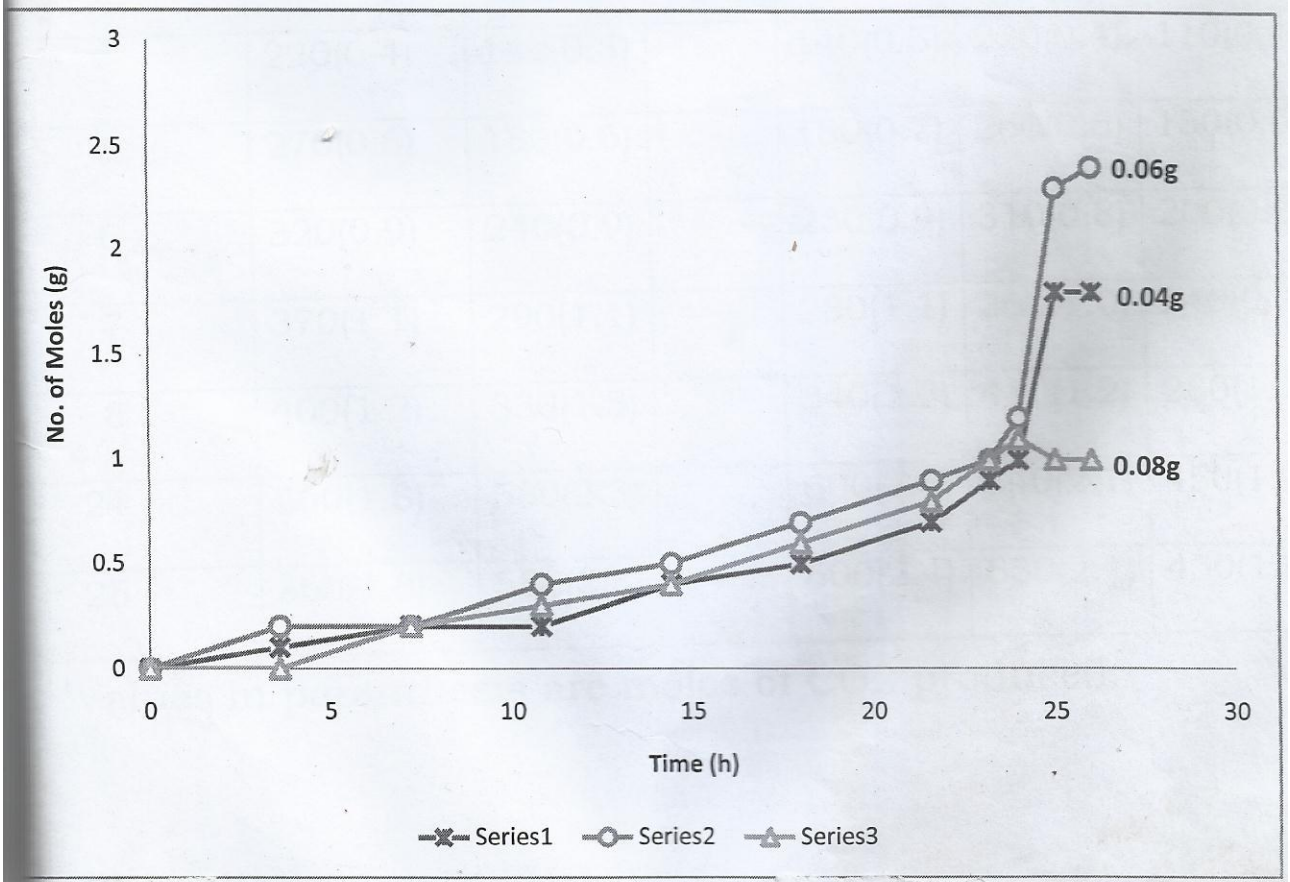
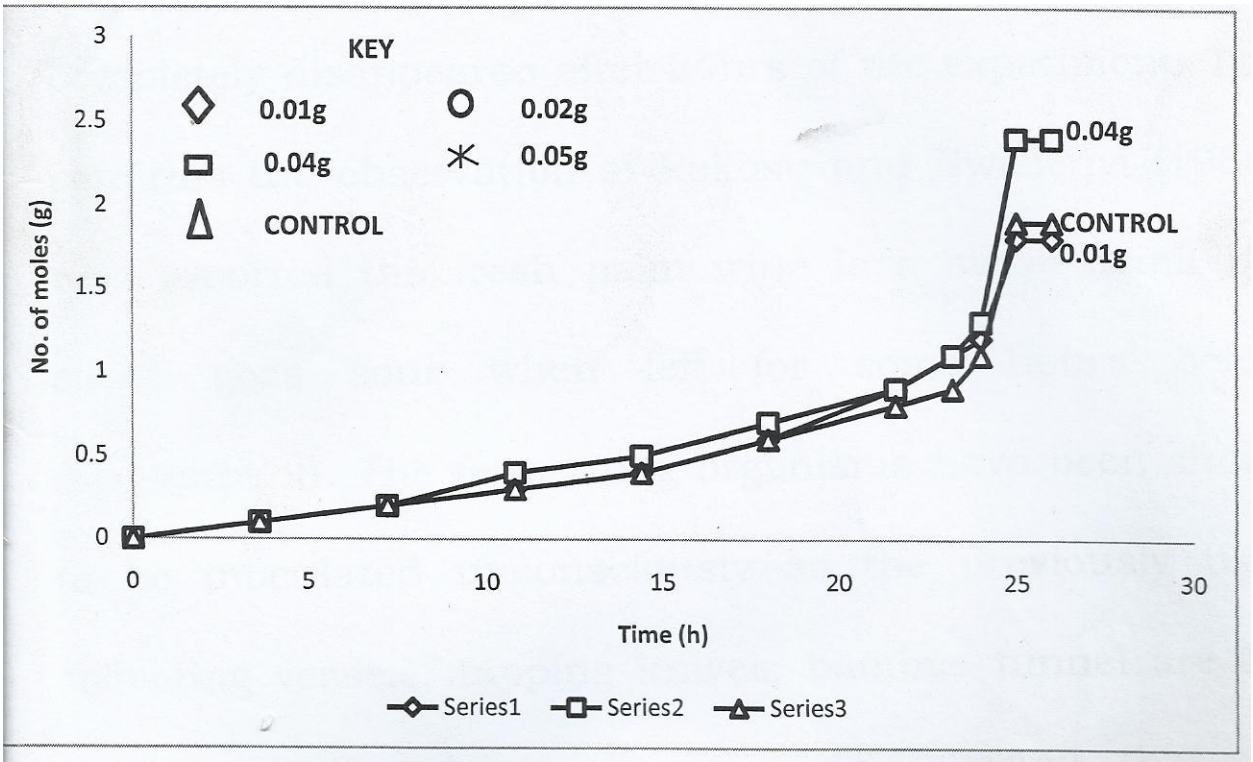


Fig 1; Fermentation rate profile of *Elaeis* sap treated with various concentration of Phyllanthus *Melleriarius* leaves 'total *flavonoids.*



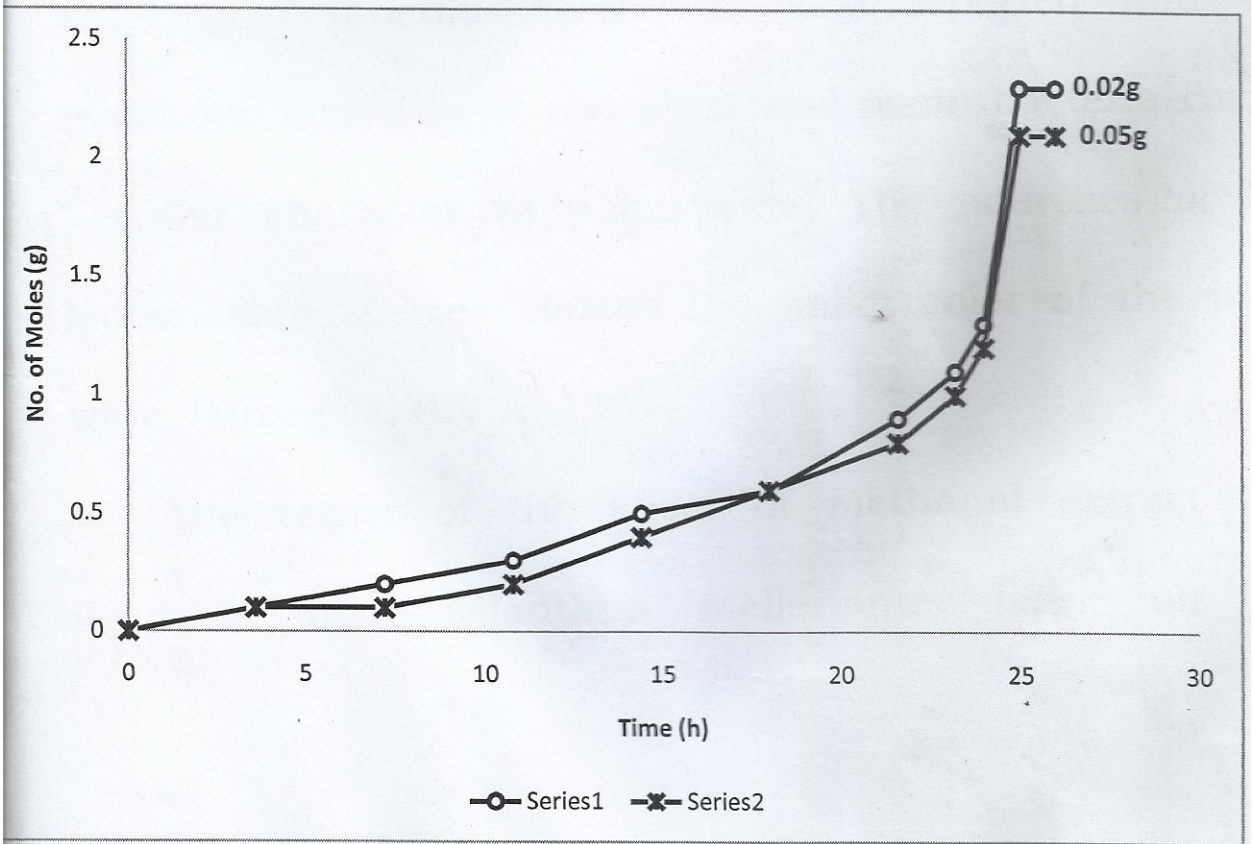


Fig 2; Fermentation rate profile of *Elaeis* sap treated with various concentration of methanol extract of *Phyllanthus Mellerianus* leaves.

TABLE 3. Fermentation rate constants of *E. guineenus* sap dosed with different concentration of 'total flavonoids' and methanol extract of Phyllanthus mellerianus leaves

Concentration (mg)	Total flavonoids	Methanol extract
10	$4.629x10^{-5}$	$3.968x10^{-5}$
20	--	$5.952x10^{-5}$
30	--	--
40	$3.268x10^{-5}$	$3.88x10^{-5}$
50	--	$3.968x10^{-5}$
60	$2.777x10^{-5}$	--
70	--	--
80	$3.704x10^{-5}$	--
Control	$5.787x10^{-5}$	$5.787x10^{-5}$

Table 4. Properties and some fermentation products of *E. guineenus* sap and wine dosed with Total flavonoids

Concentration (mg)	**Control**	**10**	**20**	**40**	**60**	**80**
Specific gravity		0.9935	0.9929	0.9911	0.9901	
Fresh sap	0.9941	--	--	--	--	--
Fermented	0.9933	--	--	--	--	--
% Alcohol % (Fresh sap)	2.0	3.0	3.0	3.5	5.0	
Fermented wine	3.0	--	--	--	--	--
Presence of methanol	-ve	-ve	-ve	-ve	-ve	-ve
Presence of Iso- propanol	+ve	+ve	+ve	+ve	+ve	-ve
Methanol extract		10	20	50	40	50
Specific gravity		0.9932	0.9915	0.9911	0.9901	0.9901
% Alcohol		2.5	3.0	3.5	4.5	5
Presence of methanol		-ve	-ve	-ve	-ve	-ve
Presence of Iso- prepared		+ve	+ve	+ve	+ve	+ve

Key: +ve = positive, -ve = negative

The observed increase in the rate of fermentation of the sap dosed with 60mg of 'total flavanoids' of *P. mellirianus* leaves might be attributed to the increased activities of microbes in the sap (Faparusi and Bassir, 1971; Okafor, 1968). It is likely that 60mg/100mL of *E. guineenus* sap is the optimum concentration of the 'total flavonoids' that activates the microbes and hence speed up the fermentation process. Figs 1 and 2 show the result obtained for the two saps, dosed with

various amount of 'total flavonoids' and methanol extract including the control. The number of moles of CO_2 liberated over time during fermentation was plotted against time (Agu et al., 2000). It was clear that the control produced the least amount of CO_2 (Fig. 2). The addition of 10-80mg of 'total flavonoid' of *P. mellirianus* affects the rate profile against that of the control (in which no 'total flavonoids' and methanol extract of *P. mellerianus* leaves were dosed). In the case of those dosed with methanol extract, the rate profile for methanol extract dosed of 10-50mg deviated slightly from those of the control. This observation might be explained from the point of view of the fact that only methanol extract contain saponin, saponin glycoside, anthracene, tannins, and flavonoids. (Table 1).

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4. CONCLUSION

This study has shown that *P.mellerianus* leaves 'total flavonoids' extract enhances the rate of fermentation and percent alcohol of *E. guineenus* sap. It has also shown that addition of methanol extract of *P. mellerianus* leaves to Elaeis sap does not influence appreciably its fermentation rate but increases the amount of alcohol produced. The study also revealed that Iso-propanol is one of the Elaeis fermentation products. And that Elaeis palm wine and those dosed with 'total flavonoids' extract and methanol extract are not toxic due to the absence of methanol as one of the fermentation product. This study has also opened fresh grounds for more research into the Biochemistry of *E. guineenus* sap fermentation for more alcohol production.

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