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Highlights

- Children below Five Years of Age
- Three Doses of Hepatitis B Vaccine
- Review: Brewing Conventional Beer
- African Plants used Against Diarrhea

Discovering Thoughts, Inventing Future

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Review: Brewing Conventional Beer with Sorghum Cultivars

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REVIEWBREWINGCONVENTIONALBEERWITHSORGHUMCULTIVARS

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Beer Brewing with Sorghum

Chikezie I. Owuama

Abstract- Malting sorghum grains yield malts with enzymes which hydrolyse their innate carbohydrates, proteins and lipids. Quality of sorghum malt is influenced by steeping regimes, steep liquor constituents, temperature and duration of germination, enzymatic activities during germination and different kilning temperature regimes. Malts of different sorghum cultivars differ in their diastatic power. Different mashing regimes influence composition of sorghum wort extracts, wort viscosity and fermentability. Fermentation conditions, yeast strains and ageing influence beer character. Sorghum beers result from fermenting either wholly sorghum wort, combinations of varying percentages of sorghum and barley wort or wort from sorghum mash treated with exogenous enzymes. Sorghum beers satisfy demand of coeliac sufferers who are allergic to gluten, present in barley beers. Current research results enhance the credibility of sorghum as sustainable substrate in conventional beer brewing. This review evaluates and updates the information on progress made at various stages of conventional beer brewing with sorghum.

Keywords: sorghum, malting, mashing, malt enzymes, diastatic power, wort, beer.

I. Introduction

Sorghum is the fifth most produced cereal in the world and belongs to the grass family, *Graminae* and tribe, *Andropogonae*. It was first used as a brewing adjunct in conventional lager beer production during the second World War (Owuama, 1999). There are two major groups of sorghum varieties viz., the non-sweet sorghum, *Sorghum vulgare* and the sweet sorghum, *Sorghum bicolor* [L] Moench), which is characterised by having sweet stalk (Owuama, 2019). Over 14,000 varieties or cultivars of sorghum exist and more new improved varieties of sorghum are being developed through continuous plant breeding research, aimed at selecting and concentrating desirable characteristics for industrial livestock feeds and food (Owuama, 1999). Among the improved varieties are those whose malts possess desirable qualities for beer brewing, such as good diastatic power, α- and β-amylase activities, proteinase activity and good extract recovery (Bekele et al., 2012; Owuama, 1999; Taylor & Daiber, 1988). The potential of sorghum as a viable alternative substrate for beer brewing, particularly in the tropics where barley does not thrive well, has been recognized (Hill & Stewart, 2019; Palmer et al., 1989; Owuama, 1999;). So far, the research on sorghum as substrate for conventional beer brewing has been going on for several decades (Hill & Stewart, 2019).

Remarkable progress has been made to date in investigating different factors that influence various stages of beer production with sorghum viz., malting, mashing, fermentation and aging (Agu & Palmer, 1996; Dale et al., 1990; Harry et al., 2019; Morall et al., 1986; Owuama, 1999). Innate enzymes in sorghum grain and those developed during malting are known to play remarkable roles in the hydrolyses of carbohydrates, proteins and lipids during mashing to yield fermentable wort (Dlamin, et al., 2015; Espinosa-Ramírez et al., 2013; Uvere & Orji, 2002). Variations in steeping, germination and kilning regimes have remarkable impact on sorghum malt quality. The mashing of sorghum malt alone or in combination with sorghum grit at varying proportions, with and without the addition of external enzymes, have also received adequate attention (Heredia-Olea et al., 2017, Hu et al., 2014). Several research results on extracts of sorghum malts and mashes (worts) reveal the presence of sugars, lipids, proteins, total soluble nitrogen and free amino nitrogen adequate to support yeast fermentation (Evans & Taylor, 1990a; Odibo et al., 2002; Okolo & Ezeogu, 1996b; Owuama, 2019; Pickerell, 1986; Taylor & Boyd, 1986;). Viscosity and fermentability of worts as well as character of sorghum beers, which include alcoholic content, specific gravity, bitterness and colour, and sensory properties (mouthfeel, appearance, bouquet, aroma and taste) have also been examined (Dale et al., 1990; Harry et al. 2019; Owuama & Okafor, 1987; Tailor & Daiber, 1988). Thus, this review, reappraises and updates the progress made so far in brewing conventional beer with sorghum

II. Sorghum Grains for Malting

Grain sorghum matures when the moisture in the grain drops to about 30 %, however, the seeds are usually too soft for harvesting when moisture content exceeds 25 % moisture. Usually, sorghum grains are harvested at optimal percentage moisture content of about 20 % so as to minimize losses and drying expense. Further drying and storage of sorghum

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however, decrease the moisture content to below 20% moisture (McNeil & Montros, 2003; Owuama, 2019). The percentage moisture content of sorghum grains for malting range from 12.5 to 20.5 % (Bekele et al., 2012; Owuama, 2019). The variations in moisture content of grains for malting may be attributable to differences in sorghum cultivar, storage conditions, maturity and age of grains (Owuama, 1999).

Sorghum grains have varying physical and biochemical characteristics within and between the two major different sorghum cultivars; *Sorghum vulgare* and *Sorghum bicolor* varieties. Sweet sorghum (*Sorghum bicolor*) varieties have larger granule size, higher water solubility index, lower amylose content and lower swelling power than grain sorghum (*Sorghum vulgare*) (Ahmed et al., 2016). Major differences between *Sorghum vulgare* and *Sorghum bicolor* is the presence of sugary stalk in sweet sorghum unlike the grain sorghum, and this may be a reflection of the physiological differences between the two cultivars (Regassa et al., 2014). Evaluation of sorghum *(Sorghum bicolor* (L.) Moench) accessions showed variations in total starch (31.01 to 64.88 %), amylose (14.05 to 23.0 %), the amylose/amylopectin ratio (0.31 to 0.73), total stalk sugar content (9.36 to 16.84 %) and crude protein (7.0 to 11.9%) (Bekele et al., 2012; Gerrano et al., 2014).

Grain characteristics usually considered for selecting sorghum variety for malting include, sorghum kernel shape and size (as reflected by thousand grain weight) (Rooney, 1973), germination energy [GE] *(measure of the percentage of grains expected to germinate fully at the time of test)*, germination capacity *(used to determine if seeds that did not germinate in the GE test are dormant or dead i.e. measures percentage of viable corns in a sample)* (Owuama, 2019), percentage moisture content and water sensitivity *(a reflection of a oxygen requirement for germination by the embryo)*. Unlike sorghum, barley contains husk, and a surface of film of water in the husk, has been shown to reduce the oxygen uptake, thereby causing embryos of water sensitive barleys to germinate to a lesser extent at low oxygen tension, thus the need for steep-aeration (air-rest or air sparging) during steeping (Crabb & Kirsop, 1969; Kelly & Briggs, 1992;). Water sensitivity of grains for malting is usually carried out to ascertain if the grains require air-rest period during steeping (Crabb & Kirsop, 1969). Thus, water sensitivity is apparently a reflection of a higher oxygen requirement for germination by the embryo. When the water sensitivity of grains for malting is less than 30 %, the grains are not water sensitive and so do not need air-rest time during steeping. Sorghum grains with water sensitivity values of 7.1 to 27.6 % have been used for malting (Anon, 1997; Davidson et al., 1976; Kelly & Briggs, 1992; Owuama, 2019). Nevertheless, no clear relationship has been established between grain moisture content and water sensitivity among different varieties of sorghum (Owuama, 2019).

Thousand grain weight of sorghum varieties used for malting differs and generally falls within 22.8 g and 58.7 g (Owuama, 2019; Subramanian et al., 1995), apparently due to varietal differences in grain sizes, storage period and conditions (Owuama, 1999; Svenson et al., 2011). The germination energy (GE) of some sorghum grains used for malting range from 96.3 to 100 % while the germination capacity (GC) falls between 99.7 and 100 % (Bekele, 2012; Dewar et al., 1995; Owuama, 2019). The recommended GE value required for sorghum to be considered suitable for malting is greater than 90% (Agu & Palmer, 2013).

III. Stages in Beer Brewing

There are fundamentally five stages in conventional beer brewing namely; malting, mashing, wort boiling, fermentation and aging. Except for wort boiling, all the other stages of the brewing process are further discussed below. Wort boiling has generally been reviewed elsewhere (Willaert Baron, 2001).

a) Malting

Malting of grains for brewing involves essentially steeping, germinating and limiting cereal seedling growth after the production of enzymes required for degradation of starch and proteins in cereal grain but before the exhaustion of polysaccharides, plus kilning or drying of green malt. Prior to malting, a small proportion of β-amylase in cereals such as wheat, rye, barley and sorghum is insoluble (Owuama, 1999; Owuama & Okafor, 1990). However, the percentage of soluble amylases in sorghum appears to be influenced by temperature and time of storage of the grains. Storing sorghum grains for 2 to 3 years at 12 to 23°C gives higher level of amylases (57 to 73%) while newly harvested grains contain about 25%. Lowering storage temperature to 7 °C reduces level of soluble amylases in the grains to about 31% after 3 years. But, storing malts for any period of time seems not to affect soluble amylase content (Owuama, 1999). Nevertheless, malting yields higher proportions of hydrolytic enzymes such as α-glucosidase, α- and β-amylases which may be either completely soluble or largely insoluble (Demuyakor & Ohta, 1992; Jayatissa et al., 1980; Taylor & Dewar, 1994). For example, insoluble amylases and α-glucosidase have been detected in malts from sweet sorghum and related variety. The insolubility of these enzymes is apparently due to their strong adhesion to insoluble malt solids (Taylor & Dewar, 1994).

Malting causes a decrease in density of caryopsis in sorghum grain (Beta et al., 1995), lowers the amount of lysine from 0.25% in unmalted sorghum to 0.18% in sorghum malt 84 and also reduces milling energy (Swanston et al., 1994). Sorghum endosperm contains both vitreous and mealy regions with the percentage of vitreous endosperm highly correlating

with grain hardness (Hallgren & Murty, 1983). Sorghum grains with intermediate endosperm texture are more suitable for malting than those with floury endosperm (Adeole, 2002). Also, waxy and hetero-waxy sorghum genotypes have soft endosperm texture which allows hydrolytic enzymes access to starch granules (with enhanced gelatinization vis-à-vis non-waxy genotypes), thus have better malting potential and consequently are more suited for beer brewing (Bekele et al., 2012; Beta et al., 2000; Taylor et al., 2006). The vitreous part of endosperm seems to contribute greatly to grain milling energy and also to malt milling energy since it is largely unmodified during malting (Owuama, 1999). Thus, there is a positive correlation between grain milling energy and malt milling energy (Swanston et al., 1992). The loss in milling energy due to starch granule modification during malting may be responsible for the highly significant correlation between diastatic power and malt milling energy. However, grain milling energy shows no significant correlation with percentage extract in sorghum (Swanston et al., 1992). Protein apparently plays a minor role in determining the quality of sorghum malt as high protein content in sorghum malt causes no brewing problems since most of the high molecular weight proteins are degraded into simpler compounds during mashing or coagulated during wort boiling and removed as protein sediment. As well, malting grains of some sorghum hybrids reduced the total phenolic content (TPC), flavan-4-ols, total flavonoid levels but more than doubled the total anthocyanin levels while the 3-deoxyanthocyanins in sorghum grains increased by about 8-fold in the malt (Khoddami et al., 2017; Owuama, 1999).

Nevertheless, malting quality of sorghum is determined by physical and biochemical factors such as temperature and time of steeping and germinating of grains with their inherent enzymic activities, kilning temperature regimes (Owuama, 1999; Owuama & Asheno, 1994), and the sorghum cultivar (Owuama & Okafor, 1987; Subramanian et al., 1995). Malt quality has been shown to influence the type and character of beer produced (Owuama, 1997). The impact of various physical and biochemical factors on various stages of malting are discussed below.

b) Steeping

Steeping involves soaking grains in water with or without air-rest until desirable moisture level (steep-out moisture) is attained. During steeping certain physical and biochemical changes occur, such as, swelling of grains, degradation of soluble carbohydrates and removal of some pigments, microorganisms and bitter substances from grains. Factors that affect the rate at which the grains absorb water include, grain structure (softer grains absorb more water than hard grains), and grain size (smaller grains absorb moisture more rapidly) (Pitz, 1989). Aeration during steeping has been shown to affect the rate at which the grain absorbs water (Olkku et al., 1991). Steeping is essentially regulated to achieve a suitable moisture level and avoid over-steeping or reaching a saturation point, which usually results in killing of seed germ. Suitable steep moisture varies with sorghum grain variety, steeping time and temperature (Owuama & Asheno, 1994; Owuama & Okafor, 1987), and steep moisture of grain directly affects sorghum malt quality (Dewar et al, 1997). Steep-out moisture contents of 32 to 35% have a positive correlation with free alpha amino nitrogen (FAN), total non-protein nitrogen (TNPN) and cold water soluble protein (CWS-P) (Ogbonna et al., 2003).

Steeping sorghum grains at temperatures of 10 to 30°C causes an increase in steep-out moisture with apparently no appreciably effect on diastatic power of malts (Owuama, 1999). Also, steeping temperature (up to 30°C) increase malt diastatic power while free amino nitrogen and extract content peak at a steeping temperature of 25°C (Oikku et al., 1991). Steep moisture affects extract yield, reducing sugar, diastatic power of malt and level of amino acids in wort. Steeping sorghum at 30°C for 18 to 22 h results in steep moisture of 44-48% which is optimal for enzymic activity (Morall et al., 1986; Owuama & Asheno, 1994; Ratnavathi & Ravi, 1991) while steep moisture of 35-40% seems to encourage rapid germination at a temperature of 22°C, in the dark (Aisien & Ghosh, 1978). Apparently, increase in steep moisture with steeping time from 12 to 20 h at 30°C is directly proportional to diastatic power of malt and consequently an increase in reducing sugar, cold and hot water extracts (Owuama & Asheno, 1994). However, steep moisture levels beyond the optimum, leads to a decrease in extract and diastatic power of malt (Owuama, 1999).

Steeping methods (i.e. with or without change of water) have virtually no effect on sorghum malt (Owuama, 1999). Steeping sorghum with increasing air rest periods of 1 to 4 h at 30°C for 48 h to attain steep moisture of 40-42%, germinating for 4 d and kilning at 50°C result in (a) a decrease in average main rootlet length (b) decrease in malting loss from 14.1-18.1% to 9.5-13.6% and (c) an increase in malt diastatic power (including α- and β-amylases) up to 3 h air-rest period followed by a decrease after 4 h. However, variations occur among sorghum cultivars e.g. the optima for α- and β-amylase activities in cultivar KSV 400 occur at air rest periods of 3 h and 1 h respectively but at 2 h and 3 h air rest periods for cultivar KSV 8 (Ezeogu & Okolo, 1995). β-Amylase activity constitutes 36-50% of total diastatic activity in cultivar KSV 400 but 27-49% in cultivar KSV 8 while cold and hot water extracts give highest values for KSV 400 and KSV 8 after air rest of 3 and 4 h respectively (Ezeogu & Okolo, 1995). Increase in steeping time plus aeration and steep water temperature enhance diastatic power. Steeping grains

plus aeration at 30°C for 40 h yield maximum diastatic power of 42.6 SDU/g. Steeping at 25°C for 40 h under air rest condition produce maximum malt FAN (119.8 mg/100g) while 24 h steeping with aeration yield highest malt extract (62.5%) (Dewar et al., 1997b). And aeration during steeping appears to enhance the extract and free amino nitrogen content of the finished malt (Dewar et al., 1997a).

Varying the duration of final warm water steep at 40°C between 1.5 h to 7.5 h and germinating for 4 d at 30°C cause (a) malting loss and a decrease in average main root length with increase in the duration of final warm water steep and (b) increase in diastatic activity, α- and β-amylolytic activities, and extract yield as the final warm water steep period increases up to 3 h and thereafter declines. However, these observations vary with sorghum cultivars (Okolo & Ezeogu, 1995b). The highest α-amylolytic activity occurs at relatively shorter duration of final warm water steep e.g. 3 h for KSV 8 and 1.5 h for KSV 400 while peak β-amylases activity result after 3 h and 7.5 h final warm water periods for KSV 400 and KSV 8 respectively. Nevertheless, diastatic activity for KSV 8 attains another peak, albeit smaller, after 7.5 h of final warm water steep, thus suggesting the involvement of at least another β-amylase component. A marked reduction in average main root length of 53% and 25% occur after 1.5 h and 3 h final warm water steep for KSV 400 and KSV 8 respectively (Okolo & Ezeogu, 1995a).

Steeping solution (i.e. water with or without amendments), time and temperature have highly significant effects on sorghum malt quality. Steeping in dilute sodium hydroxide solution enhances water uptake by sorghum grains. A positive linear relationship exists between increase in NaOH concentrations (0.1-0.6% w/v) and steep-out moisture content of grains. Steeping in 0.6% NaOH (w/v) for 48 h results in the highest steep-out moisture content of grain (Bekele et al., 2012; Beta et al., 2000). Steeping grain in NaOH (ca 0.2% v/v) and dilute formaldehyde (ca 0.05% v/v) has been shown to improve sorghum malt quality, by suppressing inhibitory effects on the malt enzymes, particularly in cultivars with high levels of condensed tannin (Beta et al., 2000; Taylor et al., 2006). Malt from grains steeped in NaOH solution vis-à-vis control malt (not steeped in NaOH), show enhanced diastatic power, free α-amino nitrogen and hot water extract (Ukwuru, 2007). In contrast, repression of carbohydrate modification occurs when sorghum grains are steeped in dilute calcium hydroxide solution (Okolo et al., 2010). Steeping sorghum continuously in alkaline liquor (0.1% NaOH) and germinating for 4 d at 30°C repress germinability (by 3-34%), root length and malting loss. However, steeping sorghum cultivar SK 5912 continuously in alkaline liquor plus a final warm water steep enhances malt diastatic activity (50-250%) and α- and β-amylase activities. β-Amylase activity constitutes over 70% of the total diastatic activity in alkaline steeped cultivar ICSV 400 malts (Okolo & Ezeogu, 1996a). In contrast, alkaline steeping of ICSV 400 with air rest and final warm water treatment repress diastatic activity by 9% although similar treatment significantly enhance diastatic power and α-amylase development in cultivars KSV 8 and SK 5912 (Okolo & Ezeogu, 1995a). Nevertheless, cultivar SK 5912 produces relatively low HWE although it has improved amylolytic activity (Okolo & Ezeogu, 1996a). As well, steeping sorghum in 0.1N ammonia solution (NH_4OH) up to 18 h increasingly reduces enzyme development, cold and hot water extracts, and malting losses (by suppressing the growth), but does not prevent mouldiness (Ilori & Adewusi, 1991).

However, soaking white sorghum grains with 1 or 2% (w/w) koji *(Aspergillus oryzae)* and germinating for 4 d yield malt with diastatic power comparable to barley malt. The addition of 1% (w/w) *A. oryzae* to sorghum grains before germination does not affect germination capacity (97.3%), whereas inoculation with 2% (w/w) reduces germination capacity by about 5%. The sorghum malts from five d of germination show similar malting losses. Addition of *A. oryzae* during malting enhances the α-amylase activity of malts but has no effect on the β-amylase activity. Addition of 1% koji during malting enhance amyloglucosidase activity (AMG) of malt while 2% koji, causes a reduction in AMG activity of the malt (Heredia-Olea et al., 2017).

c) Germination Stage

Germination basically involves outgrowth of plumule and radicle of the seedling until the production of adequate enzymes for the malt but prior to the exhaustion of seed nutrients. During seed germination, storage proteins within endosperm are hydrolysed by enzymes to provide nitrogenous compounds for grain outgrowth. Small peptides and products of partial protein hydrolysis in endosperm are translocated across scutellum to embryo where peptides are degraded by peptidases to release amino acids for plant structure and enzyme synthesis. The radicle usually grows out first before the plumule during germination. The lengths of the radicles (rootlets) and plumules (acrospires) increase with d of germination. Sorghum grains germinated for 4 d produce seedlings with radicles 2 to 5-fold longer than the plumules. Nevertheless, vegetative outgrowths in seedlings apparently have no clear relationship with the size of sorghum grains (as reflected by 1000 grain weight) (Owuama, 1999; Owuama, 2019).

Both germination period (3 to 4 d) and sorghum variety remarkably affect malt quality (Bekele et al., 2012; Owuama, 2019). Increase in germination period (2 to 4 d) show direct correlation to sorghum malt diastatic power (DP, 18.96 to 31.39 L), hot water extract (HWE, 41.85 to 85.08 %), malting loss (8.68 to 27.56 %) and

free amino nitrogen (FAN, 185.67 to 343.29 mg L^{-1}) (Bekele et al., 2012). Varietal differences and the malting processes, particularly steeping and germination influence quality of sorghum malt (Abuajah et al., 2016; Ogu et al., 2006; Svenson *et al*., 2011; Taylor et al., 2006). Germination significantly affects increase in amylase activity, malting loss, soluble solids yield and protein content (Abuajah et al., 2016; Claver et al., 2010; Svenson *et al*., 2011). As the germination period increases up to 5 d, quality of sorghum malt increases with increase in wort filtration rate, fermentable sugars, the specific gravity and wort extract but a marginal decrease in the specific viscosity (Abuajah et al., 2016).

Germinating sorghum grains at optimal temperatures of 25 to 30°C for 3 to 7 d, depending on the grain variety, leads to rapid growth of radicle, a reduction in adequate germination period and the production of well modified malts (i.e. where horny grain endosperm has completely changed to powdery, chalky state) with high diastatic power (Demuyakor & Ohta, 1992; Lasekan et al., 1995; Owuama & Okafor, 1991; Palmer et al., 1989; Ratnavathi & Ravi, 1991), hot water extract, sugar contents (Lasekan et al., 1995) and free amino nitrogen (Morrall et al., 1986). The optimal germination period varies with sorghum grain varieties and germination conditions such as, illumination and steep moisture. Three days of germination of sorghum grains steeped in the dark for 18 h, produce malts with higher diastatic power than those steeped for 32 h. As well, increasing germination period from 2 to 6 d at 30°C results in an increase in diastatic power, reducing sugar, cold water and hot water extracts (Demuyakor & Ohta, 1992; Lasekan et al., 1995; Palmer et al., 1989), as well as protein content of sorghum malt (Okoh et al., 1989). The DP increases as the germination period increased from 48 to 96 h, but no remarkable difference between 96 and 144 h. Considering the excessive malting loss and marginal increase in HWE beyond 96 h, the optimum malting period is about 96 h (Bekele et al., 2012). In contrast, germinating sorghum at relatively higher temperature of 35°C or lower temperatures of between 15 and 20°C, slows down amylase formation and invariably reduces diastatic power (Owuama, 1999; Morrall et al., 1986).

Diastatic power, which largely measures the combined activity of α- and β-amylases, is of a greater importance in sorghum malt than extract (Raschke et al., 1995) and seems to be directly proportional to its reducing sugar content (Lasekan et al., 1995). Generally, diastatic power, free α-amino nitrogen, extract and malting loss increase with germination time (Morrall et al., 1986). High moisture level in the early stages i.e. within 8 d of germination, usually results in a high diastatic power and consequently early enzymatic hydrolysis and transfer of solubilised products to embryo. The diastatic power subsequently slows down but may in some cases, increase slowly to the end of the germination period (Aisien & Ghosh, 1978; Owuama, 1999). Diastatic activity of malts range from 32.3 to 150.0 SDU/g (Subramanian et al., 1995) and over 50% of β-glucan is digested by enzymes after 2 d of germination (Ogbonna & Egonwu, 1994). However, diastatic power of 60 to 80 KDU/g is recommended for sorghum grain to be considered for commercial malting (Owuama, 1999).

Germination of sorghum grains steeped with air rest at 25-26°C for 6 d, produce malt whose percentage extract has highly significant correlation with the diastatic power (Swanston et al., 1992). Germination temperatures of 24 and 28°C are both equally good for the development of diastatic power, FAN and extract but higher temperatures are progressively worse. Germination of sorghum grains for 6 d under high (77%), medium (60.8%) and low (42.8%) moisture conditions affect the diastatic power, FAN, extract and malting loss and moisture content of green malt (Morrall et al., 1986). For example, high moisture during germination causes increases in diastatic power, FAN, extract and malting loss. However, towards the end of germination, high moisture negatively affects diastatic power (Morrall et al., 1986). A maximum diastatic power of 46.6 SDU/g occur within 5 d of germination at 24°C under medium moisture. Maximum FAN of 180mg FAN/100g malt is produced under high moisture after 6 d germination at 32°C (Morrall et al., 1986). Treatment of sorghum with thiram (0.2%) plus carbendazim (0.1%) improves seed germination by 8 to 40% and reduces seed mycoflora (Ingle et al., 1994). Sorghum grains heavily infected with mould produce malts with slightly higher amylase activity (Kumar et al., 1992), thus suggesting that fungi contribute towards the increase in amylase activity. Seed mycoflora of sorghum species include *Aspergillus flavus, Curvularia lunata, Cladosporium cladosporoides, Fusarium moniliforme, Rhizopus* sp., *Alternaria* sp., *Penicillium* sp., *Dreschlera* sp., and *Neurospora* sp. (Kumar et al., 1992; Owuama, 1991).

d) Kilning

Kilning involves the drying of green (wet and growing) malt in a kiln or oven at a relatively high temperature until the vegetative out growths become friable or brittle, desirable colour develops while the required hydrolytic enzymes for mashing remain intact. Kilning contributes to colour development which is influenced by the extent of modification, duration and levels of temperature-time sequence of kilning cycle and moisture content of green malt at different stages of the cycle (Briggs et al., 1981; Owuama & Asheno, 1994). Sorghum malts are kilned at elevated temperatures of 45 to 100°C (Owuama, 1999; Owuama & Asheno 1994), essentially to remove raw flavour of green malt and promote chemical reactions for the formation of

components which impart characteristic flavour to malt (Briggs et al., 1981). Commercially produced sorghum malts for brewing are usually dried at moderate temperatures up to 50°C (Abuajah, 2013). Kilning green sorghum malt above 50 ℃ can lead to loss of volatiles, reduced enzyme activities but enhanced malt flavour (Bekele, 2012; Dewar et al., 1997b).

Storage period of sorghum malts apparently affects the enzyme activity and the malt constituents and extracts (Etokakpan, 2004a). The diastatic power of freshly kilned sorghum malt (68.1°WK) decreases by 29% after six months of storage. Freshly kilned sorghum malt shows high wort turbidity (4.9 EBC) which drops to 0.95 EBC and 1 EBC after storage for 2 and 6 months respectively. Colour of worts derived from the malt diminishes slightly over six month-period from 7.6 EBC in freshly kilned malt to 6.8 EBC. Wort extract remains virtually unchanged throughout the six month-period probably due to the use of external amylolytic enzymes during mashing. The protein in wort extract (46.6%) decreases to 43.2% after six months. The apparent wort extract after final attenuation (AEFA) indicates more fermentability starting from two months after storage. Free-amino nitrogen (FAN) decreases from 238 mg/L to 194 mg/L after six months of storage while mash filtration period (86-93 min) using a micro-mash filter was virtually the same throughout the six months of storage (Etokakpan, 2004b).

Temperature, moisture content of green malt and duration of kilning influence amylase activity of sorghum malts (Malleshi & Desikachar, 1986). Kilning green malts with moisture contents over 10% at elevated temperature accelerates the inactivation of enzymes (Andriotis et al., 2016), but kilning sorghum green malt with less than 10% moisture at 100°C for 3 to 4 h has little effect on the amount of hydrolytic enzymes and diastatic power (Owuama, 1999). Varying kilning process produces malts of differing characteristics. Kilning malts in two stages i.e. exposing green malt initially to 55°C and subsequently to 65°C, produce malts with higher sugar content than kilning at a single temperature of 65°C (Owuama & Asheno, 1994). In two-stage treatment, initial exposure to 55°C for sometimes, considerably reduces moisture content of green malt before final temperature (65°C) treatment (Owuama & Asheno, 1994), a process which apparently encourages greater survival of hydrolytic enzymes while malt acquires characteristic flavour. Higher kilning temperature causes a relatively smaller decrease in reducing sugar and diastatic power of malts than on hot water extract and liquefying power. This is apparently due to inactivation of saccharifying amylase, β-amylase to a greater extent than liquefying amylase, α-amylase (Owuama, 1999). During kilning, reducing sugars decrease in quantity while sucrose level often increases (Owuama & Asheno, 1994) possibly because of a reversal in the action of hydrolytic enzymes (Andriotis et al., 2016) that appears not to have a direct relationship with amylase content in sorghum malt (Owuama, 1999; Owuama & Asheno, 1994) suggesting the involvement of other enzymes, with varying contributions in different sorghum varieties (Briggs et al., 1981; Owuama & Asheno, 1994).

Diastatic power and extract yield of the sorghum malt show a linear decrease with increase in kilning temperature while the total soluble nitrogen (TSN), permanently soluble nitrogen (PSN), Kolbach index and free amino nitrogen (FAN) show parabolic variation (to an optimum temperature range of 50 to 60°C) with increase in kilning temperature (Abuajah, 2013). But the colour of the worts produced from the malts dried at different temperatures show a linear increase with increase in kilning temperatures. However, the pH values of the worts did not show any significant change with increase in kilning temperature. Apparently, a temperature range of 50 to 60°C for kilning sorghum malt is suitable for producing good quality malt (Abuajah, 2013). Percentage moisture content of kilned malts from different sorghum varieties have been shown to fall between 5.2 to 13.8 % (Bekele, 2012; Etokakpan, 2004a; Ogu et al., 2006; Owuama, 2019).

IV. Enzymes in Malting

A variety of enzymes are present in sorghum grains and some are developed or activated during malting. These enzymes include; carbohydrases (α-, β- and γ-amylases), proteinases, lipases and peroxidases. Some of these enzymes present in malt are examined in greater details below.

a) Diastatic Power

Diastatic power (DP) refers to the combination of activities of enzymes (carbohydrases) in malt that hydrolyse starch into fermentable sugars. Thus, diastatic power correlates with sugar content in wort derived from mashing (Etokakpan & Palmer, 1990). Diastatic power of malt increases with steeping temperature up to 30°C and germination period up to 5 d (Dewar et al., 1996; Subramanian et al., 1995; Swanston et al., 1994) after which a plateau is reached (Okon E.U. & Uwaifo, 1985). However, brewing with sorghum *(Sorghum vulgare)* malt is apparently challenging due to low diastatic activity inadequate for complete saccharification, high starch gelatinization temperature and low FAN content (Taylor et al. 2013). Sorghum malt has a low β-amylase activity, but a higher α-amylase activity than barley malt. This leads to production of low fermentable sugars and a high dextrins content, causing an increase of viscosity (Espinosa-Ramírez et al. 2013; Owuama, 2019).

Diastatic power in sorghum malt differs with sorghum cultivars and usually comprise α-amylase and β-amylase (Mouria et al., 1998), but *Sorghum bicolor* (sweet sorghum) malt additionally contains

amyloglucosidase, thus the DP of *S. bicolor* comprises α-amylase, β-amylase and amyloglucosidase (γ amylase) (Owuama, 2019). Amyloglucosidase (γ-amylase or glucoamylase) encompasses α-glucosidase and limit dextrinase, which act synergistically with α-amylase and β-amylase respectively (Evans et al., 2010; MacGregor et al., 1999; Owuama, 2019; Preseˇki et al., 2013; Zhang et al., 2013). Generally, *S. bicolor* and *S. vulgare* varieties have virtually similar α-amylase and β-amylase activities but *S. bicolor* varieties show higher DP (Owuama, 2019; Subramanian et al., 1995). Concisely, *Sorghum vulgare* malt DP = α- + β-amylases activities while *Sorghum bicolor* malt DP > α- + β- + γ-amylases activities (MacGregor et al., 1999; Owuama, 2019; Preseˇki et al., 2013; Zhang et al., 2013;), apparently because of AMG synergism with α- and β-amylases. Malts with high levels of diastatic power are known to yield increased reducing sugar levels in wort and enhance its fermentability. Addition of AMG in mash increases diastatic power, wort glucose and total fermentable sugars equivalents (Pozo-Insfran et al., 2004) apparently due to the synergistic activity between α-glucosidase and α-amylases (Wong et al., 2007), and between limit dextrinase and β-amylases (MacGregor et al.; 1999). Diastatic power of quality sorghum malt suitable for brewing should be greater than 28 SDU/g of malt (i.e. ca 45-49 degrees Litner [°L]) (Beta et al., 1995; Taylor, 1992). Presently, malts of some sorghum cultivars have with high DP up to 136.7 °L (Beta et al., 1995; Morall et al., 1986; Owuama, 2019). Differences exist in DP of *Sorghum vulgare* cultivars and range from 112.6 to 117° while malts of *Sorghum bicolor* cultivars have DP of 123.7 to 136.7° (Owuama, 2019).

Diastatic power is measured in a variety of units viz., SABS (South African Bureau of Standards) DP assay in SDU/g (sorghum diastatic units per gram), the IoB (Institute of Brewing) in degree Lintner (°L) and EBC (European Brewing Convention) in Windisch-Kolbach (°WK) (Bajomo and Young, 1990; Etokakpan, 2004a, 1990; Owuama, 2019). SDU methods are considered suitable for sorghum DP measurements. However, in a bid to use appropriate DP unit when sorghum and barley malts are combined in one mash, it is desirable to convert SDU to °WK and °L, which are usually applied in measuring DP of barley malts, thus the need to employ the following relationships in equations, 1 to 4 (Etokakpan, 2004a)

$$SDU = 0.741°WK + 0.8272 \quad \text{Eqn. 1;}$$

$$SDU = 0.559°WK + 15.677 \quad \text{Eqn. 2}$$

$$SDU = 1.6397°L - 1.0506 \quad \text{Eqn. 3;}$$

$$SDU = 1.06°L + 19.748 \quad \text{Eqn. 4}$$

As well, DP in °WK can be converted to °L by using the equation below (Hopkins et al., 1934);

$$°L = (°WK+16)/3.5 \text{ i.e. } °WK = 3.5 \times °L - 16 \quad \text{Eqn. 5}$$

Diastatic power of sorghum varieties determined in different units show a range of 56 to 132 °WK corresponding with 29 to 67°L and 47 to 87 SDU (Etokakpan, 2004a). Equations 1 to 4 are applicable under appropriate conditions (Etokakpan, 2004a). The IoB and EBC methods are considered suitable for sorghum DP measurements.

b) Alpha Amylase

Alpha-amylase (endo-acting) randomly hydrolyses starch chains at α(1,4) glucosidic linkages distant from the ends of the chains and from α(1,6) linked branches in the chains yielding dextrins, oligosaccharides, maltose and glucose (Briggs et al., 1981; de Souza & Magalhães, 2010). During malting significant quantity of α-amylase is produced in embryos of sorghum (Palmer, 1989). α-Amylase in sorghum malt may be either completely soluble or largely insoluble depending on variety of sorghum (Demuyakor & Ohta, 1992; Jayatissa et al., 1980). The formation of α-amylase requires adequate oxygen, however this can be prevented in the presence of excess carbon dioxide (Owuama, 1999). α-Amylase activity in sorghum malt is 25 to 183 SDU/g depending on sorghum variety (Aisien & Ghosh, 1978) and increases with sorghum diastatic power in cultivars with SDU values greater than 30 (Lasekan et al., 1995; Ratnavathi & Ravi, 1991). Differences exist in α-amylase activities of malts between sorghum cultivars *S. bicolor* ([sweet sorghum] and *S. vulgare* [non-sweet sorghum], and within the various sorghum cultivars (Owuama, 2019; Subramanian et al., 1995). Generally, α-amylase activities of different *S. bicolor* cultivars (71.8-83.2°) are slightly lower than those of *S. vulgare* malts (78.8- 85.2°). α-Amylase activities in *S. vulgare* varieties are 70-75 % of the diastatic power (DP) and substantially higher than the 56 - 61 % of DP in *S. bicolor*. However, α-Amylases activities in both *S. bicolor* and *S. vulgare* malts are 2 to 4-fold those of β-amylases. In *S. bicolor*, α-amylases activities are 3.6 to 5-fold those of amyloglucosidase (AMG) (Owuama, 2019; Subramanian et al., 1995).

Steeping sweet sorghum grains at three different time intervals of 8, 12 and 16h and germinating subsequently for 2 and 3 d show the highest amylase activity (1266.10 μg of protein/15 min/g) and highest reducing sugars (33.85 mg/g) in 16h steeped grains, germinated for 3 d. Similarly, addition of different concentrations (0.1, 0.5 and 1%) of commercial α-amylase (Palkozyme), show the highest reducing sugar value (78.83 mg/g) at 1% enzyme concentration at 70°C for 24h (Mesta et al., 2018). However, alkaline steeping with final warm water steep improves substantially α-amylase activity in sorghum malt in sorghum cultivar SK 5912 but represses it in cultivars ICSV 400 and KSV 8. The reason for this variation with different cultivars is unclear but may be attributable to α-amylase polymorphism. It is known that steeping or germinating

conditions influence the inhibition or enhancement of the synthesis of particular isoforms detectable in cereal grains during malting (Jones & Jacobsen, 1983; Owuama, 1999). The inhibition of a specific dominant α-amylase isotype by native proteinaceous α-amylase inhibitor in sorghum (Macgregor & Daussant, 1981) invariably depresses total amylase activity while inactivation of the inhibitor during alkaline steeping enhances total amylase activity (Okolo & Ezeogu, 1996a). Alternatively, enhancement of alkaline α-amylase activity in one cultivar but not in another may be attributable to the capacity of alkaline steep liquor to influence protein-binding properties of tannins/ polyphenols which vary in concentration and distribution in various sorghum cultivars (Chavan et al., 1981). Tannins (located mainly in pericarp and testa) and other polyphenols can bind to proteins including enzymes, and are therefore likely to inactivate enzymes involved in hydrolysis of endosperm materials (Chavan et al., 1981; Owuama, 1999).

c) Beta Amylase

Beta-amylases (exo-acting) hydrolyse penultimate α-1,4 glucan linkages from the non-reducing end of starch yielding maltose and beta-limit dextrins. Non-germinated sorghum grain show virtually no β-amylase activity (Taylor & Robbins, 1993). Sorghum β-amylase develops during germination by transforming from a latent bound form to a free or active form in starchy endosperm (Aisien & Palmer, 1983; Owuama, 1999),. β-Amylase may be either completely soluble or largely insoluble in malt depending on the variety of sorghum (Agu & Palmer, 1997; Demuyakor & Ohta, 1992; Jayatissa et al., 1980; Owuama, 1997). Malts made from sweet sorghum and related variety, birdproof kaffircorn usually contain insoluble amylases which appear to adsorb tenaciously to insoluble substances, thus making aqueous extraction impossible (Owuama, 1999). Thus, peptone solutions have been used to liberate the bound β-amylase, resulting in higher DP of the sorghum malts in coloured and bird-proof varieties (Agu & Palmer, 1996; Kumar et al., 1992; Owuama, 1999). However, a contrary report indicates that β-amylase is not bound since neither reducing agents nor papain treatment affects its activity (Taylor & Robbins, 1993). Apparently, the difference in observations reflect variation in physiological activities of the sorghum cultivars. Beta amylase activities in malts vary with sorghum cultivars and in *S. vulgare cultivars range from* 22 – 25 % of DP and slightly higher than 19 – 22 % of DP in *S. bicolor* (Owuama, 2019). β-Amylase activity in sorghum malt range from 11 to 41 SDU/g (Beta et al., 1995; Taylor & Robbins, 1993) and constitutes 27 to 49% of total diastatic activity in sorghum (Ezeogu & Okolo, 1995).

β-Amylase is more labile than α-amylase and is influenced by germination time and temperature. A rapid increase in β-amylase activity occurs within the first 2 d of germination and subsequently declines in rate of increase up to 6.5 d. β-amylase activity is inversely related to temperature, giving the highest activity at 24°C over a range of 24 to 32°C (Taylor & Robbins, 1993). More maltose producing enzyme, β-amylase is present in sorghum malts made at 25°C and 30°C, producing 66% more maltose during mashing than malts made at 20°C (Owuama, 1999). There is a wide variations regarding β-amylase activity of sorghum malt and this may be due to the assumptions that β-amylase activity is the difference between total amylase activity and α-amylase activity. An assumption which ignores activities of other starch degradation enzymes such as α-glucosidase and limit dextrinase.

β-Amylase activity also shows significant correlation with malt diastatic power and is completely inactivated in 15 min at 68°C (Taylor & Robbins, 1993). However, alkaline steeping with final warm water steep treatment and air rest result in a decrease in β-amylolytic activity in cultivar ICSV 400 but an increase in both cultivars KSV 8 and SK 5912 (Okolo & Ezeogu, 1996a). The reduction in β-amylase activity in cultivar ICSV 400 may reflect repression of the synthesis of a major β-amylase isotype. Isoelectric focussing indicates that sorghum β-amylase has a major and a minor isoenzyme of approximate pl 4.4-4.5 (Taylor & Robbins, 1993). β-Amylase heterogeneity is influenced by malting stage and conditions (Laberge & Marchylo, 1986; Macgregor & Matsuo, 1982). The activity of β-amylase in sorghum malt significantly increases when a combination of final warm water and air rest cycles are employed during malting. β-amylase activity of malt is known to be prominently affected by steep regime, alkaline steep liquor, and kilning conditions as well as their various interactions. Steeping in $Ca(OH)_2$ enhances malt β-amylase activity at higher kilning temperature (50°C) unlike steeping in KOH that shows a reduced effect. Nevertheless, the extent of β-amylase activity enhancement is cultivar dependent (Okungbowa et al., 2002).

d) Amyloglucosidase or Glucoamylase (γ-Amylase)

Amyloglucosidase or glucoamylase [γ-amylases] is exo-acting and hydrolyses both α-1,4 and branching α-1,6-linkages to yield glucose. Amyloglucosidase comprises α-glucosidase and limit dextrinase. α-Glucosidase and limit dextrinase have been shown to act synergistically with α-amylase and β-amylase respectively, in starch hydrolysis, yielding glucose (Evans *et al*., 2010; MacGregor *et al*., 1999; Owuama, 2019; Presečki *et al*., 2013; Zhang *et al*., 2013). Evaluation of sorghum cultivars reveals that while amyloglucosidase is present in *S. bicolor* cultivars, it is not detectable in *S vulgare* varieties (Owuama, 2019). Malts of *S. bicolor* cultivars show

amyloglucosidase (AMG) activities ranging from 14.5 – 21.3°. The α-amylase activities in *S. bicolor* cultivars are 3.6 to 5-fold those of AMG, while the β-amylase activities are 1.2 to 1.9 fold those of AMG. AMG activities in *S. bicolor* malts are 12-16 % of their diastatic power (DP) (Owuama, 2019). Generally, DP in *Sorghum vulgare* malts are equal to α-amylase plus β-amylase activities, but in *Sorghum bicolor*, DP is greater than the sum of α-amylase, β-amylase and AMG activities, thus suggesting synergism among the amylases (Owuama, 2019). See below for discussion on α-glucosidase and limit dextrinase.

e) Alpha-Glucosidase

Alpha glucosidase or maltase is one of the enzymes involved in starch degradation during cereal seed germination (Sun & Henson, 1992). α-Glucosidase in germinating grains catalyses hydrolysis of terminal, non-reducing α-(1, 4) glucosidic linkages in both oligosaccharides and α-glucans yielding glucose (Andriotis et al., 2016; Owuama, 1999;). α-Glucosidase in sorghum malt contributes to glucose production in wort by hydrolysing terminal α-1,4 linked D-glucose residues to release glucose (Agu & Palmer, 1997). Purified alpha-glucosidase is quite thermolabile (less than 50°C), cleaves a single glucose from a starch chain or splits maltose to produce two glucose units, thus reducing the level of maltose in the fermentable sugar profile (Fox, 2018). Although, α-glucosidase in sorghum is soluble in water, it is also active in insoluble state while adhering strongly to insoluble malt solids (Taylor & Dewar, 1994; Watson & Novellie, 1974). α-Glucosidase development in sorghum in influenced by germination period and temperature. Limited α-glucosidase extracted with sodium chloride under alkaline conditions is enhanced by adding papain (Owuama, 1997). Sorghum malt from 5 d germination at 30°C, show highest α-glucosidase activity in extract with sodium phosphate pH 8 containing L-cysteine at pH 3.75 compared to those of 1 to 4 d (Agu & Palmer, 1997; Taylor & Dewar, 1994). The sorghum malt with the highest α-glucosidase activity however produces the lowest glucose levels in wort, suggesting that α-glucosidase is not the dominant glucose-producing enzyme during mashing of sorghum malts (Agu & Palmer, 1997). Malts from germinating sorghum at 30°C show the highest levels of α-glucosidase, β-amylase and α-amylase as well as the highest maltose to glucose ratio, relative to 20°C and 25°C germinated sorghum malt. However, the role of each enzyme in the sugar ratios is unknown (Agu & Palmer, 1997). Nevertheless, the sorghum malts produced at 20°C and 25°C yield worts which contain more glucose than worts from malts produced at 30°C. The individual activities of α-glucosidase, α-amylase and β-amylase of sorghum malts apparently do not correlate with the sugar profile of the worts (Agu & Palmer, 1997). However, α-glucosidase is known to have synergistic activity with α-amylase in solubilizing starch (MacGregor et al., 1999).

Mashing at pH 4, near optimum for α-glucosidase yields relatively higher proportion of glucose than at usual mash pH 5-5.5, which is optimal for β-amylase (Taylor & Dewar, 1994). Although, sorghum malt α-glucosidase activity is highest at pH 3.75, it is still quite active at pH 5.4 employed in mashing sorghum malt (Agu & Palmer, 1997). However, at pH 5-5.5, both total fermentable sugars and free glucose increase with mashing temperature to a maximum at 70°C but the proportion of glucose declines with increasing mashing temperature from 58.6% at 60°C to 23.1% at 80°C. In contrast, mashing at pH4 produces less amount of total fermentable sugars and free glucose at 70°C than at 60°C (Taylor & Dewar, 1994). Maltose in sorghum worts produced at 65°C is limited because of inadequate gelatinization of starch and not β-amylase and α-amylase activities since gelatinization of the starch granules of sorghum malt occurs between 68-72°C (Taylor and Taylor, 2018). Hence, the decantation mashing method yielded sorghum worts with high levels of maltose, particularly when sorghum malt is produced at 30°C (Agu and Palmer, 1997). Higher amount of glucose is observed in wort from EBC conventionally mashed malt as against using pre-cooked malt insoluble solids where α-glucosidase inactivation occurs preventing hydrolysis of maltose to glucose and resulting in high maltose levels in sorghum worts (Taylor & Dewar, 1994).

f) Limit dextrinase

The activities of starch degrading enzymes (including α-amylase, β-amylase, alpha glucosidase and limit dextrinase) result in the production of a mixture of low molecular weight dextrins (Aisien et al., 1983; Etokakpan & Palmer, 1990; Okon & Uwaifo, 1985; Taylor & Robbins, 1993). Limit dextrinase (LD) also called R-enzyme, pullulanase, isoamylase or amylopectin 6-glucanohydrolase, is a debranching enzyme that hydrolyses α-(1 → 6) linkages in amylopectin or in branched dextrins derived from the actions of α- or β-amylases (Yang et al., 2009). LD cleaves the α-1,6 branches on amylopectin, producing linear α-(1 → 4)-linked chains for α- and β-amylases to further hydrolyse to glucose and maltose. The degree of branching on amylopectin and amylose in any cereal used either as malt or as an adjunct source, could impact on the residual dextrins which are not fermentable (Denyer et al., 1999). Purified limit dextrinase from malted sorghum flour readily hydrolyses alpha-limit dextrins which have maltosyl or maltotriosyl side-chains, pullulan, amylopectin and beta-limit dextrin (Haedi et al, 1976). Though, LD is quite temperature sensitive, it can survive for a reasonable time in mash, where it cleaves α-1,6 linkages and thereby contributes remarkably to

fermentable sugars (Fox, 2018; Hu et al., 2014; Izydorczyk & Edney, 2003). The initial temperature of the brewing process influences LD activity, and with highly branched amylopectin, more non-fermentable solubilized residual dextrins are produced that affect beer flavour and contribute to mouthfeel (Langstaff & Lewis, 1993). Maintaining optimum temperature of 60-62°C for malt limit dextrinase as opposed to 50°C of purified LD, and lowering pH from 5.8 to 5.4 increase wort fermentability due to increased LD activity. However, wort fermentability is more strongly correlated to free LD activity of malt than to α- and β-amylase activities (Stenholm & Home, 1999). Nevertheless, limit dextrinase has been shown to have synergistic activity with β-amylase in solubilizing starch (MacGregor et al., 1999).

Dextrins containing from 4 to 10 glucose units have been observed in sorghum malt, wort and beer. During 10 d malting period, about 5% fermentable sugars and trace amounts of dextrins are detectable. Using maize adjunct during mashing at pH 4, produce a wide range of dextrins which greatly diminish towards the final stages of mashing. Both sorghum and barley beer contain similar amounts of dextrins, majority of which are branched, and the activity of LD largely reduce their concentration (Glennie & Wigh, 1986).

g) Carboxypeptidases and Proteinases

Carboxypeptidases (exopeptidases) and proteinases (endopeptidases) are important in protein mobilisation during grain germination. Peptidase formation requires adequate oxygen but is prevented in the presence of excess carbon dioxide (Owuama, 1997). Carboxypeptidases specifically hydrolyse solubilised proteins to free alpha amino nitrogen (FAN) [proteolytic breakdown products of endosperm proteins comprising amino acids and small peptides], which is the source of nitrogen essential for anabolic functions of germinating seedling and as nutrients for yeast metabolism in wort (Baxter, 1981; Enari & Sopanen, 1986). Germination conditions and sorghum cultivar influence carboxypeptidase activity. For example, carboxypeptidase activity increases with germination time up to 4 d showing 4 times the activity in resting grains (Evans & Taylor, 1990a). Also moisture, temperature and germination time significantly affect carboxypeptidase activity with the highest activity occurring in malt from 4 d germination under medium moisture at 24°C, and yielding maximum FAN value of 275μg FAN/5h/g dry malt (Evans & Taylor, 1990a; Morrall et al., 1986). Sorghum malts resulting from different final warm steep treatment periods show poor correlation between the period of final warm steep treatment and carboxypeptidase activity, whose levels vary with sorghum cultivars. Also, correlation between sorghum malt FAN and carboxypeptidase activity can be poor or strong depending on cultivar (Okolo & Ezeogu, 1996b). Proteolytic enzyme activity in sorghum is influenced by both cultivar and malting conditions but steeping does not significantly affect proteinase or carboxypeptidase activity. However, different sorghum cultivars grown and malted under similar conditions differ significantly in proteinase (endopeptidase) and carboxypeptidase activities (Evans & Taylor, 1990b).

Germination temperature (24-32°C) and moisture have little or no effect on proteinase activity (Evans & Taylor, 1990b). Germinating sorghum for 36 h or 48 h causes a considerable increase in protease activity in embryo or endopeptidase activity in both embryo and endosperm (Morrall et al. 1986). Increase in germination time up to 4 d moderately increase proteinase activity with a maximal yield of 1604μgN/5h/g dry malt. The highest proteinase activity differs with sorghum malts resulting from different final warm steep period and also with various cultivars (Okolo & Ezeogu, 1996b). Proteinase activity in cultivar ICSV 400 rises from 1224 to 1469μgN/3h/g dry malt as final warm steep period increases from 1.5 to 3.0 h. However, proteinase activity declines with increase in final warm steep period beyond 3.0 h suggesting an optimum final warm steep period similar to that for carboxypeptidase activity. Nevertheless, sorghum cultivar, KSV 8 attains highest proteinase and carboxypeptidase activities at 6 h final warm steep period (Okolo & Ezeogu, 1996b).

Optimal proteinase and carboxypeptidase activities occur after 3 h final warm water steep period in cultivar ICSV 400 but after 6 h final warm water steep in cultivar KSV 8 (Okolo & Ezeogu, 1996b). However, higher proteinase activity occurs in cultivar KSV 8 in relation to cultivar ICSV 400, although with lower CWS-protein in KSV 8. This apparent contradiction can be attributed to qualitative differences in complexity and structure of endosperm proteins of various sorghum cultivars and/or differences in the nature of the major proteinase isoforms in grains (Okolo & Ezeogu, 1996b; Riggs et al., 1983). Apparently, the highest proteinase and carboxypeptidase activities occur in the same final warm water treatment period for given sorghum cultivars (Okolo & Ezeogu, 1996b). Varying sorghum cultivars and air rest periods from 1 to 4 h during steeping with 6 h final warm water (40°C) steep, greatly influence CWS-protein, total cold water soluble, cold water soluble protein modification index, total free alpha amino acid nitrogen, and carboxypeptidase and proteinase activities of malt (Okolo & Ezeogu, 1995b).

Evaluation of the effects of calcium ion in steep liquor, on sorghum endosperm reserve protein mobilization of two sorghum cultivars, ICSV 400 and KSV 8, reveal remarkable enhancement of total non-protein nitrogen (TNPN) accumulation in ICSV 400 malt, but 23 to 69% repression in KSV8 malt. Likewise, Ca^{2+} ion treatment effectively stimulates peptide accumulation in ICSV 400 indicating that it largely

enhances TNPN accumulation in this cultivar unlike in KSV 8 where peptide accumulation is highly repressed. Protein solubilisation, soluble protein accumulation and cold water soluble protein modification in ICSV 400 and KSV 8 cultivars were highly repressed by Ca^{2+} treatment. Ca^{2+} treatment remarkably stimulates carboxypeptidase development in both cultivars, slightly enhances proteinase development in KSV 8 but causes reduced proteinase development in ICSV 400 (Okolo et al., 2011).

White non-tannin sorghum grain produces substantially higher levels of FAN than white type II tannin sorghum, due to the presence of tannin. Incubating sorghum grains with combined exogenous neutral proteinase and amino-peptidase, improve FAN production. However, malts from the white non-tannin and tannin sorghum types produce similar FAN levels when incubated in the absence of the exogenous proteases. Malts of both tannin and non-tannin sorghums incubated with neutral proteinase alone yield substantially more FAN (124-126 mg 100 g^{-1}) than the grains (61-84 mg 100 g^{-1}). The combination of amino-peptidase and proteinase do not improve on FAN yield. Also, malting does not influence wort free amino acid profile. Nevertheless, group B amino acids constitute the highest percentage (42-47%) (Dlamin et al., 2015).

h) Lipases

Lipase (triacylglycerol acyhydrolase) catalyses the hydrolysis of triacylglycerides to free fatty acids and glycerol (Lin et al., 1983). Malt lipoxidase catalyses peroxidative reaction that converts free fatty acids to hydroperoxides and aldehydes which have detrimental effects on beer such as poor acceptability and reduced shelf-life (Kobayashi et al., 1993). A higher level of fatty acid is present in sorghum relative to barley, wheat and millet (Osagie, 1987). Sorghum grains contain detectable lipase activity which varies slightly during 24 h steeping period at 30°C and increases during germination to about 4-fold after 96 h. However, lipase activity varies among different sorghum (red and white) cultivars, but peaked in malts derived from 4 d of germination, though the red showed higher activity (Nwanguma et al. 1996, Uvere & Orji, 2002). Differences in lipase activity apparently suggest variations in lipase synthesis or differences in endogenous regulators of lipase activity (Chapman, 1987). The lipase activity in plumule, endosperm and radicle are 68%, 29% and 3 % respectively in 72 h old malt. Sorghum malt lipase apparently consists of three isoforms, two of which have their highest activity optima within the acidic pH range (Uvere & Orji, 2002). The optimal pH for sorghum lipase is 7 although the activity range is between pH 5.5 and 9. The percentage lipase activity at pH 5.5, 6, 8 and 9, relative to that at pH 7 are 50%, 95%, 88% and 60% respectively (Nwanguma et al. 1996; Uvere & Orji, 2002). Because of the wide pH range, sorghum lipase activity occurs during steeping, malting and mashing (Gram, 1982, Uvere & Orji, 2002). Lipase activity decreases in sorghum malt after kilning at 48°C for 24 h to between 24% and 66% of total lipase activity in green malt depending on sorghum variety, however mashing at 65°C yields wort with no detectable lipase activity (Uvere & Orji, 2002). Exposing malt crude water extract for 10 min to temperatures of 50°C, 60°C and 65°C reduce lipase activity to 57%, 43% and 14% respectively, of the original activity and total loss of lipase activity result from heating extract for 30 min at 50°C (Nwanguma et al. 1996).

i) Peroxidases

Plant peroxidases are heme-proteins that utilise hydrogen peroxide ($H_2O_{2)}$ to oxidise various hydrogen donors including phenolic substances, amines, ascorbic acid, indole and particular inorganic ions (Diao et al., 2011; Dicko et al., 2006; Dunford, 2010; Murphy et al., 2012). Peroxidase catalyses the reductive destruction of hydrogen peroxide and invariably contributes to the defence system of living organism against peroxidation of unsaturated lipids involving oxygen radicals (Floyd, 1990; Nwanguma & Eze, 1995). Lipid peroxidation causes reduction in quality and shelf life of most cereal products. Peroxidase activity in different sorghum varieties differs with malting regimes. Various sorghum varieties differed in their expression of peroxidase over different germination periods. The least peroxidase activity was ≤ 0.6 peroxidase units in the different varieties, occur at the end of 24 h steeping period. The highest peroxidase activity (above 6 peroxidase units) occur between 72 and 96 h of germination. Generally, the size of the sorghum grain affects peroxidase expression. Most of the sorghum varieties that show remarkable differences in peroxidase expression between the raw grains and the green malt at the end of germination period, are among the smallest sized varieties (Nnamchi et al., 2013).

Lipid peroxidation is undesirable in malting and brewing (Bamforth et al., 1993; Kobayashi et al., 1993). During malting, aldehydes and other lipid peroxidation products are released that affect the availability of wort nutrients, interfere with yeast metabolism, cause flavour deterioration and affect colloidal stability of beer (Bamforth et al., 1993; Nnamchi et al., 2013). Peroxidase activity increases by about 14-fold during the germination of sorghum grains steeped at 30°C for 24 h, however the levels present vary with sorghum varieties (Nwanguma & Eze, 1995). Peroxidase activity of 39-40% is detectable in endosperm while a combined activity of 56-61% occur in the acrospire and rootlet. The optimal pH for sorghum peroxidase is 5.5 and kilning at 48°C for 24 h shows no depressing effect on the peroxidase activity (Nwanguma & Eze, 1995). In crude extract, sorghum peroxidase activity decreases from 77% to 7.5% after 15 min exposure to temperatures of 60°C to

80°C respectively. Nevertheless, peroxidase activity declines to 5% in 5 min at 85°C and is completely absent at higher temperatures. Sorghum peroxidase survives better in wort than crude extract and about 50% of peroxidase activity is retained in wort after mashing for 1 h at 65°C (Nwanguma & Eze, 1995). Since remarkable amounts of lipid oxidation products form during mashing (Meersche et al., 1983), it is therefore important that sorghum peroxidase remains active in wort to remove oxygen radicals at the later stages of brewing.

V. Malting Loss

Malting loss is the summation of leaching/steeping, metabolic/respiration and vegetative/sprout losses (Malleshi & Desikachar, 1986; Owuama, 1999). Basically, it is the loss in weight of grains after malting. However, malting loss in commercial kaffircorn malts are only due to metabolic and leaching losses, since roots and shoots are not usually removed but milled in with the berry (Owuama, 1997). Factors which influence malting losses include germination period, germination temperature, steep moisture, kilning temperature and sorghum variety. Malting losses, generally vary with germination temperature and increase with germination period. Percentage malting loss increases with germination period among sorghum varieties and range from 8.68% to 27.56% (Bekele, 2012). Malting loss is higher at 25°C (8.4%) and 30°C (10.9%) than at 20°C (6.5%) and malts produced at 30°C over 1 to 6 d show losses of 3 to 31% depending on sorghum variety (Owuama, 1999; Beta et al., 1995; Owuama & Asheno, 1994). Germination temperatures of 25 to 30°C are optimal for amylase and diastatic power development in sorghum malt, and encourage vigorous respiration and high malting losses (Owuama, 1999). High steep-out moisture of grains and watering during malting, enhance the rate of germination and malting loss while reducing malting loss by lowering temperature or moisture level causes a marked decrease in diastatic power (Beta et al., 1995; Owuama, 1999). Thus, the attainment of a good diastatic power in sorghum malt may be linked to high malting loss. Percentage malting loss has also been shown to differ among sorghum varieties and generally lower among cultivars of *Sorghum bicolor* (16.3 and 17.8 %) than those of *Sorghum vulgare* (16.4 to 26.0 %) (Owuama, 2019). A respiration/metabolic loss of 10 to 15% and percentage vegetative loss for *S. bicolor* cultivar (8.9 - 10.1%) and *S. vulgare* varieties (7.2 - 13.3 %) are expected in well-malted sorghum with good diastatic power (Owuama, 2019). Minimizing malting loss, while achieving sufficient grain modification during malting is desirable to produce malt for brewing (Aisien et al., 1983; Bekele, 2012; Ezeogu & Okolo, 1996).

VI. Proteins in Sorghum Grains and Malt

Amorphous storage proteins associate with starch granules within endosperm of barley and sorghum, and during grain germination, malt proteolytic enzymes initiate the modification of grain reserve in endosperm by hydrolysing proteins associated with starch granules, thereby exposing the starch and increasing its susceptibility to amylolysis (Holmes, 1992; Palmer, 1989). The hydrolysis of insoluble reserve protein in germinating grain provides amino acids necessary for the synthesis of hydrolytic enzymes and grain structural materials in growing tissues of seedling (Owuama, 1999). Nevertheless, malts show lower protein than unmalted grains and malts from sorghum cultivars with high diastatic activity exhibit high levels of albumin-globulin fraction (Subramanian et al., 1995). Crude protein contents of grains differ with sorghum varieties and range from 7.0 to 12.3% (Bekele et al., 2012; Owuama, 2019).

During malting, FAN is mainly derived from the hydrolysis of proteins in the endosperm and comprises free amino acids and small peptides, produced by proteinases and carboxypeptidases activities of the malt, and remarkable portion of the nitrogen in the kernel is transferred to the roots and shoots. Proteolytic activity increases with germination time during malting (Evans and Taylor, 1990a). FAN increases in wort with germination period (48-144 h) is partly due to the inclusion of dried roots and shoots (which are rich in FAN) during mashing. The addition of dried roots and shoots of sorghum malt during mashing to ensure adequate FAN level in the wort is necessary particularly for cultivars with minimal FAN content (Dewar, et al., 1997a). Unlike barley malt which is much richer in proline, sorghum malt has asparagine and glutamine as its two most important free amino acids. Also, sorghum malt has higher percentage of amino acids readily assimilated by yeast than barley malt and other cereals such as wheat (Hill and Stewart, 2019). However, percentage malt total nitrogen in sorghum malts vary considerably between 2.0 and 3.1 % while their protein contents range from 12.2 to 19.5 % (Owuama, 2019).

Sorghum malts obtained by steeping grains for 22 h followed with 4 h air rest and further 24 h wet steep at 20°C (giving steep moisture of 34-35%) and subsequently germinated for 5 d at 20°C, 25°C and 30°C show more effective hydrolysis of endosperm proteins at 20°C than at 25°C and 30°C. Malting at 30°C transfers larger quantities of nitrogen from endosperm to embryos (axes and scutella) than malting at 20°C and 25°C, but less amino acids and peptides are transferred to root during malting at 30°C than at 20°C and 25°C. Nitrogen may also move from root to embryo by physiological mechanisms (Agu & Palmer, 1996).

Steeping regime and sorghum cultivar significantly influence FAN values. Generally, exposing sorghum grains to a steep regime incorporating air rest cycles and final warm water steep result in the highest FAN level in ICSV 400 and KSV 8 varieties while continuous steep regime without final warm water steep produce the lowest FAN values. Cultivar and duration of final warm water (40°C) steep highly influence protein modification indices viz., soluble protein of cold water extract (CWS-protein), total non-protein nitrogen (TNPN), a small peptide accumulation, free alpha amino nitrogen, carboxypeptidase and proteinase activities (Okolo & Ezeogu, 1996b). The application of final warm water steep without air rest stimulates FAN development in cultivars ICSV 400 and KSV 8 but significantly represses FAN development in SK 5912. Nevertheless, significant improvement of FAN values occurs in all sorghum varieties after the application of air rest cycles during steeping although the FAN levels vary with cultivar (Ezeogu & Okolo, 1996). Apparently, these differences reflect variations in grain protein structure and degradability (Riggs et al., 1983), amino acid transport processes, and probably differences in enzyme characteristics (Owuama, 1999).

Generally, ICSV 400 shows higher FAN, CWS-protein solubilising activity and accumulation, and better protein modification potential than KSV 8. However, lower TNPN and TNPN-FAN difference in ICSV 400 contrasts with its high FAN, thus suggesting superior anabolic protein turnover apparently from efficient peptide translocation process. Nevertheless, the levels of nitrogenous substances are inconsistent with the proteolytic activities suggesting the involvement of factors other than proteolysis in protein modification (Okolo & Ezeogu, 1996b). Remarkably, KSV 8 records lower FAN although it generally expresses higher carboxypeptidase activity in relation to ICSV 400. This suggests a variation in the rate of protein synthesis from FAN and thus a possible higher rate of anabolic protein turnover in KSV 8 and lower FAN accumulation (Okolo & Ezeogu, 1996b).

Four days of germination of sorghum cultivars steeped in alkaline liquor (0.1% NaOH solution) for 48 h at 30°C under different steeping regimes, reveal that steep regime, steep liquor and sorghum cultivar highly and significantly influence the protein modification indicators viz., CWS-protein, CWS-protein modification index, TNPN, peptide accumulation, FAN, endo- and exo-protease activities. Alkaline steeping causes a highly significant increase in sorghum malt FAN (Okolo & Ezeogu, 1996b). FAN in malt is a net balance of amino acids and peptides resulting from storage protein degradation and those utilised for synthesising new proteins in roots and shoots of growing plant (Morrall et al., 1986; Taylor & Boyd, 1986). FAN development vary among cultivars probably because of differences in major enzyme characteristics and rate of protein metabolism during sorghum grain malting as well as variations in grain protein structure and degradability (Riggs et al., 1983), amino acid and peptide transport processes (Owuama, 1999). Nevertheless, other miscellaneous cultivar-dependent factors also play a role in the control and modulation of protein degradation and synthesis in germinating plant seeds (Shutov & Vaintraub, 1987). Free alpha amino nitrogen development in malt is important in brewing as it constitutes about 70% of total FAN in wort (Pickerell et al., 1986; Taylor & Boyd, 1986).

In general sorghum malts from grains steeped with air rest period and steepout moisture of 33-35% reveal increase in diastatic power, FAN, extract and malting loss with germination time. Germination temperatures of 24 and 28°C are equally good for the development of diastatic power, FAN and extract. Diastatic power, FAN, and extract and malting loss increase with high moisture during germination (Morrall et al., 1986). Germination at 32°C under high moisture shows similar FAN level in malt at 3.0-4.5 d, possibly a period of catabolic and anabolic equilibrium, before increasing further to a maximum of 180 mg FAN/100 g malt after 6 d (Morrall et al., 1986).

FAN levels in sorghum grain wort mashed with commercial enzymes are considerably lower than those obtained with sorghum malt (Dale et al., 1989; Goode et al., 2003). FAN levels of 130–150 mg/L are considered adequate to support optimal yeast growth and fermentation efficiency (Dhamija & Singh, 1978; O'Connor-Cox & Ingledew, 1989), thus to overcome the very low FAN levels when brewing with sorghum, high levels of proteolytic enzymes are required. Use of reducing agents such as 2-mercaptoethanol (Dale et al., 1990; Hamaker et al., 1987), sodium bisulphite and ascorbic acid (Aisien & Palmer, 1983; Arbab and El Tinay, 1997) have been shown improve sorghum protein hydrolysis. Addition of reducing agents such as KMS (potassium metabisulphite), when mashing sorghum grain with exogenous protease also improves FAN production. The rate of sorghum protein hydrolysis is significantly increased by KMS which reduces intermolecular molecular disulphide bonds in the kafirin polymers and oligomers, and apparently allows better access of protease to the kafirin (Ng'andwe et al., 2008). Presumably, reducing agents can reduce the stabilizing inter- and intra-molecular disulphide bonds, which influence the conformation of kafirin before and after exposure to wet cooking (Enari & Sopanen, 1986; Ng'andwe et al., 2008).

VII. Water Extracts of Malts

Hot water extracts (HWE) and Cold water extracts (CWE) (which *are soluble products from enzyme hydrolysis within endosperm during the malting process*

that include sugars and amino acids) vary with sorghum cultivars. However, there are substantial differences between CWE and HWE of malts among various sorghum cultivars (Holmes, 1991; Owuama, 2019). HWE values have been shown to be about 1.5 to 3 fold higher than CWE in both *Sorghum bicolor* and *S. vulgare* varieties. CWE apparently correlate with total nitrogen and protein contents in malts from *S. bicolor* but not with those from *S. vulgare* (Owuama, 2019). CWE and HWE are influenced by cultivar, steeping conditions and steep liquor. CWE is generally enhanced in certain cultivars by alkaline steep with final warm water steep but depressed in others apparently due to alkaline steep repression of certain malt properties like diastatic power and α-amylase activity (Okolo & Ezeogu, 1996a). A combination of air resting and final warm water steep at 40°C reduces kernel growth and malting loss but significantly improves CWE, HWE, diastatic power, α- and β-amylase activities. But final warm water steep without air resting causes a decrease in extract recovery and enzyme activity (Ezeogu & Okolo, 1994). Generally, sorghum malt produced at 25°C and 30°C show depressed HWE yield and total soluble nitrogen development during mashing in contrast to that produced at 20°C (Agu & Palmer, 1996). Steeping sorghum grains in alkaline liquor generally enhances HWE of malts in cultivar ICSV 400 but reduce HWE in cultivar SK 5912 albeit with an increase in α- and β-amylolytic activities. This suggests possible inhibition of other enzymes contributing to endosperm cell wall structure solubilisation such as exo-and endo-proteases and β-glucanase, and consequent prevention of amylase access to starch granules for efficient conversion (Okolo & Ezeogu, 1996a).

The α-amylase development in sorghum malt is better enhanced during germination at 30°C than at 28°C. Using infusion mashing, hot water extract (HWE) show remarkable difference within germination time over 3–6 d, but not influenced by germination temperature. However, using the decantation mashing method, no appreciable change in HWE occurred over the germination period. Relatively, low HWE obtained from sorghum malt in the infusion mashing process indicate that it is unsuitable for optimal extract production from malted sorghum. Sorghum malt from germination at 28°C releases more FAN products into the worts than the malt from 30°C, using both the infusion and decantation methods (Ijasan, et al., 2011).

Generally, malting increases water extract (WE), water extractable protein (WEP), HWE, and hot water extractable protein (HWEP) of sorghum grains by 3.0-, 3.4-, 2.3- and 2.0-fold respectively (Subramanian et al., 1995). Diastatic activity correlates significantly and positively with WEP and water-extractable contents of malt produced at 30°C. Percentage WEP as a proportion of total protein vary between 11.0 and 36.0% and HWEP range from 19.3 to 44.1% (Subramanian et al., 1995). CWS-protein in grains steeped with aeration at 30° and final warm water steep at 40°C for 6 d is significantly higher than those steeped without air cycle. This may be due to an increase in protein solubilisation in response to improved enzyme synthesis or better hydration of endosperm and enzymes mobility (Ezeogu & Okolo, 1996). The CWS-protein yield varies with sorghum cultivar in both protein solubilisation activity and CWS-protein accumulation. For example, CWS-protein value from cultivar SK5912 (1680 mg % dry malt) is significantly higher than those for ICSV 400 (1030 mg % dry malt) and KSV 8 (1280 mg % dry malt) (Ezeogu & Okolo, 1996).

VIII. Mashing

Mashing in conventional brewing is basically by two methods, viz., decoction and infusion processes (Briggs et al., 1981). During mashing, water soluble substances dissolve, enzymes hydrolyse solubilised starch and proteins and to a lesser extent other higher molecular weight substances essential for the type and character of beer, and finally dissolved substances are separated. Hydrolyses of substances involve enzymes such as amylases, proteases, peptidases, transglucosidases and phosphorylases which are regulated by factors like temperature, pH, time and concentration of the wort. Mashing extracts about 80% of the dry matter from the malt while cold water extracts about 15% (Briggs et al., 1981; Mandl & Wagner, 1978).

Mashing sorghum malt by decoction process and infusion methods are influenced by temperature-time regimes and sorghum variety, and produce worts of varying composition (Owuama & Okafor, 1987). In three-stage decoction, about 70% of mash is boiled to gelatinise starch for greater amylolytic activity while creating plenty of opportunity for proteolytic enzyme action and minimising scope for the development of lactic acid bacteria (Owuama, 1999). Sorghum starch gelatinization temperature (68-72℃) is influenced by kafirin (sorghum prolamin protein) (Taylor and Taylor, 2018). Kafirin resistance to protease digestion (mainly due to intermolecular disulphide bonding), affects the digestibility of starch. (Elkonin, et al., 2013), resulting in partial starch hydrolysis into fermentable sugars (Heredia-Olea, 2017). Thus, starch digestion by amylolytic enzymes increase the quantity of protein in individual kafirin fractions (α, β and γ kafirin) and reduce the amount of high molecular weight proteins. And consequently, kafirin digestion by pepsin results in the formation of polypeptide (Elkonin, et al., 2013). Mashing of sorghum malt at 65°C and 70°C for 30 min each, at second and third stages respectively, of three stage-decoction process, provides wort with complete hydrolysis (Owuama, 1999; Solomon et al., 1994). A longer incubation time at saccharifying temperature

(65°C) than dextrinising temperature (70°C) gives wort with higher reducing sugar levels (Owuama & Okafor, 1987). However, maintaining mash for 60 min at second stage and 70°C for 60 min in third stage produce more fermentable sugars (Owuama & Okafor, 1987). Reducing sugars and proteins in wort increase as concentration of sorghum malt rises from 15 to 25% (Owuama & Okafor, 1987), apparently because of a simple increase in mash concentration and stability of enzymes. Infusion mashing at 65°C releases higher levels of peptides but lower quantities of α-amino nitrogen and total soluble nitrogen than decantation mash in which decanted enzymatically active wort is used to mash gelatinised sorghum starch at 65°C (Mandl & Wagner, 1978; Owuama, 1999).

Mashing sorghum malt by the European Brewing Convention (EBC) congress procedure (EBC, 1987), which involves hydrolysis of pre-cooked malt insoluble solids using an enzymatic malt extract, yield wort with approximate maltose to glucose ratio of 4:1. But mashing malt extract without pre-cooking of malt insoluble solids produce worts containing approximately equal amounts of maltose and glucose (Taylor & Dewar, 1994). Nevertheless, both treatments give the same quantity of total fermentable sugars and wort extract. Infusion mashing of 13.8 dry weight of total cereal content, {composed of 21% sorghum malt (diastatic power ca 38 SDU/g) with cooked adjunct of 70% maize grit and 8% sorghum malt}, at 60°C, pH 4 for 2 h in the presence of about 200 ppm calcium ions results in almost complete conservation of diastatic activity, increase in extract, maximum yield of reducing sugar in wort, and the detection of α-amylase activity which appears to be lacking in the absence of calcium ions (Taylor & Daiber, 1988).

A relatively high level of starch extracts and low level of fermentable extracts have been obtained by using a non-conventional mashing procedure i.e. decanting active enzyme wort after mashing sorghum malt at 45°C for 30 min, and gelatinising starchy grist residue at 80-100°C before mixing with wort, to achieve a saccharifying temperature of 65°C (Palmer, 1989). Palmer (1989), attributed the result to smaller quantities of β-amylase in the wort. Lower wort filtration volume is produced in mashes containing raw sorghum than in all malt mashes. Adding external enzyme during mashing of sorghum malt increases extract yields and free amino nitrogen in wort (Agu et al., 1995; Bamforth et al., 1993). Introducing industrial enzyme preparations containing α-amylase and β-glucanase to mashes with raw sorghum yield higher values of extract recovery in relation to untreated mashes. Addition of amyloglucosidase (AMG) to sorghum during mashing results in an improved wort yield, filtration rate, and a higher percentage ethanol after fermentation (Urias-Lugo and Saldivar 2005, Espinosa-Ramírez, 2014). Moreover, adding enzyme preparations containing a neutral proteinase increases wort total nitrogen and free amino nitrogen while enzyme preparations with β-glucanase or cellulase decrease wort viscosity relative to untreated mashes (Dale et al., 1990). Also a 20% (w/v) sweet potato flour substitution for sorghum malt increases maltose level in wort, apparently because of the presence of β-glucanase (limiting in sorghum) in sweet potato (Etim & Etokakpan, 1992). Mashes composed of 50% malt and 50% raw sorghum and supplemented with enzyme preparations show an increase in wort filtration volume relative to similar mashes without enzyme supplements (Dale et al., 1990). Mashing 50% malt and 50% polished (whole) sorghum by single decoction mashing regime produce wort with filtration behaviour (lautering) comparable to that from control mash (70% malt and 30% maize grits) while wort produced by double mashing regime from 20% malt and 80% raw sorghum supplemented with industrial enzyme show slow filtration and result in sweet and turbid wort. Apparently, this reflects low malt content of grist and lack of suitable material to form mash filter bed (Dale et al., 1990).

a) Wort and Wort Extracts

Worts are usually produced from mashing malts plus adjuncts and contain a variety of fermentable extracts. Worts from two varieties of sorghum malts mashed using commercial brewing enzymes reveal sorghum wort and evaporated wort (extract), containing sufficient sugars and amino acids required for yeast growth and alcohol production during fermentation (Odibo et al., 2002). Mashing different varieties of sorghum malts with exogenous enzyme extracts from sweet sorghum (*Ipomoea batatas*) and yellow yam (*Discorea cayensis*) yield worts containing higher reducing sugars than the untreated malts. However, worts from malts mashed with *Discorea cayensis* show remarkably higher reducing sugars than those mashed with *Ipomoea batatas* (Owuama & Adeyemo, 2009). Worts from barley malt and waxy sorghum grits are comparable to commercial wort and provide adequate substrates for *Saccharomyces cerevisiae* fermentation (Barredo Moguel et al., 2012). Sugar profile of wort from sorghum malt, barley malt, sorghum and barley grains mashed with commercial enzyme show that wort of barley malt and sorghum malt have similar ratios (1:7) of glucose to maltose. However, mashing barley or sorghum grains with commercial enzymes alter the glucose to maltose ratio in both worts, although a greater change is observed in wort from sorghum grains. Nevertheless, hydrolysis with commercial enzymes yield more glucose in sorghum wort, but have more maltose in barley wort. Adding barley malt to sorghum grains mashed with commercial enzymes, re-establish the glucose to maltose ratio in sorghum mash (Okolo et al., 2020).

Worts from grists containing raw sorghum are of higher fermentability and show lower levels of total nitrogen and free amino nitrogen compared to control worts. Worts from mashes containing raw sorghum and malt comprising 20% malt and 80% raw sorghum possess higher levels of total nitrogen and free amino nitrogen than is expected from the reduction of malt content of mash, consistent with the release of nitrogenous components (polypeptides, peptides and amino acids) from sorghum in wort. Wort from 20% malt and 80% raw sorghum has greatly reduced total nitrogen and free amino nitrogen compared to that of all malt wort (Dale et al., 1990). However, levels of both total nitrogen and free amino nitrogen in wort from 20% malt and 80% raw sorghum are not reduced in proportion to malt content of mash, thus suggesting that nitrogenous materials from sorghum are released during mashing into wort. The wort from 20% malt and 80% raw sorghum contains higher proportions of aspartic acid, serine, asparagine, glutamic acid, alanine and histidine but lower proportions of proline, leucine and phenylalanine than control wort (Dale et al., 1990). Worts derived from sorghum malt-1% koji (sorghum grains steeped with 1% *Aspergillus oryzae* and germinated for 4 d) using double mashing procedure generated 27% more fermentable sugars and 24% more FAN. Remarkably, wort from sorghum-1% koji malt contains 8.8% less fermentable sugars compared to the barley malt. However, barley wort has higher maltose concentration than the sorghum worts. The sorghum-2% koji malt does not yield more fermentable sugars than sorghum-1% koji malt. Sorghum malt and sorghum malt-1% koji produced 12°P worts with 40% and 21% less fermentable sugars respectively, compared to the control wort from barley malt (Heredia-Olea et al., 2017).

Worts from upward infusion mashing contain more reducing sugars and proteins than those from downward infusion process. Perhaps, initial high temperature (70°C) of downward infusion method inactivates some saccharifying and proteolytic enzymes (Owuama & Okafor, 1987). Worts from three-step decoction and upward infusion mashing processes contain virtually the same quantities of reducing sugars and proteins although mashing malt of different sorghum varieties with three mashing processes, yield worts with little variation in the types of sugars present (Owuama & Okafor, 1987). Mashes with grists containing high proportions of raw sorghum (50-80% malt replacement) yield high values of extract and produce worts of lower nitrogen, free amino nitrogen, viscosity and colour but higher pH values than in worts from all malt mashes (Dale et al., 1990). Increase in the proportion of raw sorghum in grist relative to malt results in decline in extract recovery, wort total nitrogen, free amino nitrogen but increase in pH. Also, worts from mashes containing raw sorghum have lower viscosity than those from all malt worts (Dale et al., 1990). Mashing of grists containing 50% extruded whole sorghum produces worts of high yield and low viscosity. Increasing the proportion of extruded sorghum in grist causes a decrease in wort filtration volume, total nitrogen and FAN (Dale et al., 1989). The wort filtration behaviour of mashes containing sorghum extruded at 175°C compare favourably with all malt control and is superior to those of mashes containing sorghum extruded at 165°C or 185°C. The results are comparable to those with extruded barley and extruded wheat as brewing adjuncts (Dale et al., 1989).

Generally, mashing sorghum malt, with three-step decoction, upward and downward infusion mashing methods yield worts with similar amino acids. The amino acid, tryptophan which seems to be absent in sorghum grain (Aisien et al.,1983) is present in worts from sorghum malt (Owuama & Okafor, 1987). Except proline, amino acids in wort are assimilated by yeast during fermentation and preferentially provide nitrogen for yeast growth while their metabolic products affect beer flavour and stability (Owuama, 1999). However, yeasts can also utilise some small peptides which only permit slow growth (Bamforth, 2001) thus emphasising the importance of high level of free α-amino nitrogen (FAN) in wort to support rapid and proper fermentation (Owuama, 1999). Mashing at 51°C and pH 4.6 yield approximately 30% free amino nitrogen (FAN) essential for yeast growth during fermentation while the rest 70% is pre-formed in malt and adjunct (Taylor & Boyd, 1986). And, sorghum beer contains low percentage of proline indicating good quality FAN (Taylor & Boyd, 1986). In infusion mashing at 60°C, pH 4.0 for 2 h, very high (VH) or high medium (HM) FAN worts promote almost complete attenuation of sugars in 48 h while low FAN worts require 72-96 h. High FAN worts promote more rapid fermentation of available sugar by yeasts than low FAN worts and a highly significant correlation exist between total brewing time and total soluble nitrogen in wort (Agu et al., 1995; Pickerell, 1986;). The higher the initial FAN concentration, the greater the rate of uptake by yeast (Jones & Pierce, 1969). Further, wort sugar level which influences overall demand for FAN seems not to affect FAN uptake rate (Pickerell, 1986). FAN in wort is higher after 120 h than after 24 h, particularly in high FAN wort. This may be attributable to lysis of aging or dead yeast cells and nitrogenous substances excreted by yeast cells during fermentation (Pickerell, 1986). Higher initial FAN level encourages greater rate of ethanol production, thus, in very high FAN wort, ethanol production is slightly faster than in medium high FAN wort, indicating possible FAN optimum for sorghum beer fermentation. Furthermore, in very low FAN wort, fermentation is protracted and sugar utilisation by yeast is poor and invariably alcohol yield is low. However, sugar uptake depends on its level in wort i.e. high wort sugar is taken up faster than low wort sugar (Pickerell, 1986).

Proteolytic activity during infusion mashing at 60°C and pH 4.0 for 2 h produces about 30% of wort FAN while 70% is pre-formed in malt and adjunct. FAN in sorghum beer wort is good as it contains a low percentage (ca 10%) of proline (Taylor & Boyd, 1986). Optimum mashing conditions for FAN production are 51°C and pH 4.6. Raising the ratio of sorghum malt to adjunct leads to a proportional increase in wort FAN while raising ratio of adjunct to malt results in a decrease in wort FAN. However, wort FAN is directly proportional to malt FAN and the addition of microbial proteolytic enzyme to mash increases wort FAN (Taylor & Boyd, 1986).

IX. Fermentation and Beer Characteristics

Yeast is usually pitched into wort, which consists mainly of fermentable sugars, including glucose, fructose, sucrose, maltose and maltotriose, as well as dextrins, nitrogenous materials, vitamins, ions, mineral salts, and trace elements (Bamforth, 2001). During fermentation, brewing yeasts adapt quickly to the wort environment, utilizing available nitrogen for the synthesis of cellular proteins and other cell components (Hill & Stewart, 2019). Wort encourages the growth of new yeast cells which ferment the medium to produce ethanol, carbon dioxide and other metabolic products, many of which contribute to the flavour of the beer (Ferreira & Guido, 2018). Beer brewed from the normal wort of sorghum is lighter in colour than that brewed from the re-dissolved sorghum extract (evaporated wort). The lower alcohol values or higher colour of beer brewed with sorghum extract was linked to the Maillard reaction, which occurs during the process of evaporating the wort to produce the extract. However, organoleptic assessment showed that beer brewed using the extract was generally acceptable. (Odibo et al., 2002).

Fermentations of lager worts from waxy sorghum grits inoculated with either yeast cultured in wort or yeast grown in yeast-malt media produce levels of alpha amino nitrogen (AAN) and fusel alcohols comparable to that of commercial wort. The oxygen concentration decrease from 20% at the start of fermentation to below 1% after 72 h fermentation reflecting a gradual change from aerobic to anaerobic condition. The utilization of AAN from waxy sorghum grits wort for production of amyl-isoamyl alcohol, propanol and isobutanol is comparable to the control barley wort, over 144 h of fermentation. The isobutanol produced has the least concentration. Propanol production started after 24 h fermentation of worts inoculated with yeast cultured in wort, and after 36 h with yeast cultured in yeast-malt media. The concentration of ethanol and fusel alcohols in sorghum beer falls within the commercial beer range (Barredo Moguel et al., 2012).

Worts from grist containing extruded sorghum ferment more quickly than all malt wort and attain lower final gravity values (Dale et al., 1989). Worts and beers produced under isothermal infusion mashing conditions from grists comprising 70% malt plus 30% extruded sorghum and 100% malt filter without difficulty. Beers from grists containing extruded sorghum contain lower levels of total nitrogen and FAN compared to all malt beer, an observation which is consistent with extruded sorghum contributing little or no nitrogenous material to wort and beer (Dale et al., 1989). Beers from grists containing extruded sorghum are of sound flavour and show reasonable foam stability behaviour (Dale et al., 1989). Fermentation of normal brewing sorghum wort produced slightly higher levels of alcohol than evaporated sorghum wort (extract) (Odibo et al., 2002). However, the non-fermentable residual dextrins are solubilized during brewing and remain in beer and contribute to mouthfeel (Langstaff and Lewis, 1993).

Beers produced from 50% malt and 50% polished sorghum, and 20% malt and 80% raw sorghum filter without difficulty and have sound flavour (Dale et al., 1990). Beers produced from 50% malt and 50% polished sorghum contain lower levels of isobutanol, 2-methylbutanol, dimethylsulphide and higher level of n propanol and diacetyl in relation to control beers. The post-fermentation gravity, colour and pH are comparable to control beers (Dale et al., 1990). Carbohydrate composition of beer brewed from 20% malt and 80% raw sorghum compare favourably with those from all malt beer as well as that from commercial beer brewed from 60% malt and 40% sorghum grits. However, form stability behaviour of beer brewed from 20% malt and 80% raw sorghum is poor relative to that from all malt beer (Dale et al., 1990).

The polypeptide content of beer influences foam stability behaviour and susceptibility to non-biological haze development (Dale & Young, 1987). The low total nitrogen content of beer from 20% malt and 80% raw sorghum is responsible for high resistance to non-biological haze formation but low head retention. Beer susceptibility to microbial spoilage may be influenced by level of free amino nitrogen present (Owuama, 1999). Thus, low levels of total nitrogen and free amino nitrogen in beer from 20% malt and 80% raw sorghum may confer good storage properties against non-biological and microbial spoilage (Dale et al., 1990).

Supplementation of sorghum mash comprising sorghum malt plus adjunct (regular or waxy sorghum) with β-amylase or amyloglucosidase and using a double-mashing procedure yield sorghum malt worts with increased amount of fermentable sugars. Addition of amyloglucosidase during mashing increases total sugar content by 20% and glucose content by five-fold

vis-à-vis worts without exogenous enzymes. Worts from barley malt and sorghum malt contain adequate quantity of free amino nitrogen. Fermentation of worts by typical lager brewing conditions yield barley malt beer containing approximately 1% more ethanol relative to the sorghum malt beers that are not supplemented with exogenous amylolytic enzymes. Fermentation of worts from AMG supplemented mash produce beers with ethanol increase by 1.1% units, and comparable contents regardless of the type of malt. Fusel alcohol concentrations do not differ with mash treatments. Addition of amyloglucosidase to mash is known to give higher yields of alcohol in 100% gluten-free sorghum beers (Espinosa-Ramirez et al., 2013). Addition of β-amylase or amyloglucosidase (AMG) (Urias-Lugo and Saldivar, 2005), during mashing of sorghum malt, results in improved wort yield and filtration rate, as well as a higher percentage of ethanol production in beer. However, alcohol content of sorghum beer is approximately 1.1% less than barley malt beer. Introduction of AMG during mashing has no effect on colour, pH and FAN content of wort (Cela et al., 2020)

European beers brewed with sorghum generally yields beer with lower alcohol contents than barley beers. Lager beers produced using worts adjusted to 15° Plato from sorghum malt and inoculated with 1% *Aspergillus oryzae* yield 21.5% more volume than sorghum malt wort and 5% more than wort from barley malt. The major fermentable sugar in all worts is maltose. Higher amounts of glucose are present in both sorghum worts vis-à-vis barley malt worts (Rubio-Hores et al., 2020). Beer from sorghum malt–*A. oryzae* wort has similar specific gravity and alcohol content compared to the barley malt beer. Sorghum malt–*A. oryzae* beer contained lesser amounts of hydrogen sulphide, methanethiol, butanedione, and pentanedione relative to barley malt beer. Sorghum malt–*A. oryzae* lager beer shows similar yield for wort extract and alcohol content compared to the barley malt beer but varies in key volatiles, colour and aromatic compounds (Rubio-Flores et al., 2020).

Gluten is a protein found in most grains commonly used in brewing beer including barley, wheat, rye and oats. Barley malt contains traces of hordein (gluten), thus, barley beer contains gluten too high to be safely consumed by those suffering from coeliac disease (Tanner et al., 2013). Therefore, grains which lack gluten such as, corn, rice, sorghum, buckwheat, millet and quinoa, are suitable for brewing gluten–free beer. Presently, sorghum malt which lacks gluten has proven to be an excellent substrate and is currently used to produce gluten-free beers acceptable to sufferers of celiac disease (allergy/intolerance to gluten) (Hager et al., 2014).

x. Sorghum as Adjunct

Sorghum was recognised as an important adjunct in brewing during World War II (Owuama, 1997). Brewing adjuncts are essentially starchy materials with little or no protein content. They are a potential source of additional alcohol and may add to the colour, taste, aroma, vitamin, protein content and head retention of beer (Briggs et al., 1981; Dhamija & Singh, 1978). Other unmalted materials such as bajra, tapioca (*Manihot esculentum*), soy beans, wheat, maize and barley flours have also been added to grists as adjuncts (Agu, 2002; Dale et al., 1989; Dhamija & Singh, 1978).

Sorghum grain composition, properties, morphology and anatomy have been reviewed (Ogbonna, 1992; Owuama, 1999). In grain sorghum, there are both soluble and insoluble amylase fractions (Owuama & Okafor, 1990). The insoluble amylases which adhere tenaciously to insoluble substances still remain active in certain varieties of sorghum and are solubilised by breaking the link through a prolonged grain protease action during aqueous extraction. However, the activity of grain amylases varies with sorghum variety and are apparently involved in hydrolysis during mashing. Optimal temperatures for β-amylase (60-65°C) and α-amylase (72-75°C) in grain sorghum differ slightly from one variety to another while optimal pH of the enzymes fall between 5 and 6 (Owuama, 1999; Owuama & Okafor, 1990).

Contradictory reports on the necessity to gelatinise starch adjuncts for amylase to act (Agu & Palmer, 2013; Elkonin et al., 2013; Ezeogu & Okolo, 1996) has been attributed to differences in fineness of grinding, thickness of mash or quantity of enzymes (Briggs et al., 1981). However, mashing gelatinised sorghum grits, at different proportions with barley malts produce worts of varying contents (Owuama, 1999) while adding an industrial enzyme, "thermamyl", used by Nigerian breweries for mashing unmalted sorghum, increases yield of extract in wort when combined with malt (Agu et al., 1995). The introduction of external enzyme to 100% gelatinised sorghum malt during mashing produces lager beer comparable to commercial brands obtained from barley malt (Olatunji et al, 1993). Nevertheless, a 40-70% substitution level of sorghum for barley malt is considered adequate for brewing lager beer with virtually the same organoleptic properties as beer produced with only barley malt (Dhamija & Singh, 1978; Ogbonna & Obi, 1992; Owuama, 1999). Utilization of sorghum adjunct, at 5 to 20% level, showed a progressive decrease in extract recovery, solubilisation of nitrogen, and production of free amino nitrogen and peptide nitrogen in the wort. Sorghum adjunct has been shown to release higher levels of FAN and peptide nitrogen in extracts than barley adjuncts, a difference that may influence fermentation potential of the wort (Agu, 2002).

Brewing grits from four different decorticated sorghum genotypes, brown normal (BNO), white normal (WNO), white waxy (WWX) and white hetero-waxy (WHWX) show that decorticated kernels have lower protein, crude fibre, ash, and colour values and higher starch contents than their respective whole kernels. The extract yield of brewing adjuncts from decorticated BNO, WNO, WWX and WHWX were 81%, 87.4, 89.9, and 90.0 respectively. Worts from WWX brewing adjuncts filter faster than the hetero-waxy, white normal and brown normal. Worts from all the sorghum genotypes standardized to 14°P, show similar viscosity, α-amino nitrogen, pH and colour values. White sorghums with hard and waxy endosperms are most suited for use as brewing adjuncts (Osorio-Morales, et al., 2000). Sieving analysis of some sorghum grains as well as their hot water extractable (HWE), hot water extractable protein (HWEP) and free amino nitrogen (FAN) show that cultivars with high starch and amylose contents plus low protein and fat percent will make better adjuncts based on their HWE and HWEP yields. However, the suitability of sorghum variety as brewing adjunct for lager beers is apparently not determined by the grain size (Ratnavathi et al., 2000).

Fermentation of wort from all barley malt (ABM) mash and commercial enzyme/barley malt/sorghum adjunct (CEBMSA) mash of similar wort gravity reveals similar glucose to maltose ratios and similar amino acid spectra. ABM yields 27% more glucose and 7% more maltose than CEBMSA. After yeast fermentation, ABM mash produce 9.45% alcohol by volume (ABV) while the commercial enzyme/barley malt/sorghum adjunct mash produced 9.06% ABV (Okolo et al., 2020).

XI. Conclusion

Variations in physical and biochemical characteristics of sorghum cultivars, steeping solution without or with amendments such as ions and koji, *Aspergillus oryzae*, as well as temperature and period of germination influence optimal malting conditions and eventually malt quality. Consideration of a reasonable number of malting variables are necessary for selecting proper sorghum malt for brewing beer. Equally essential are optimising conditions for mashing and fermentation of worts to achieve the expected goal of producing sorghum beer comparable to barley beer. The wort filtration problem encountered from brewing with sorghum may be resolved by using the filter press instead of lauter tun and artificial husks from nylon materials of plant tissue (Owuama, 1999).

However, the distinct differences that exist between the structure and physiology of the aleurone, embryo and starchy endosperm cells of sorghum and barley grains (Aisen & Palmer, 1983; Palmer, 1989; Palmer et al., 1989), questions the expectation of producing similar character of lager beer from the two different grains. Also, disparities in their malt characteristics, such as β-glucan and pentosan levels, as well as amino acid profiles of malt worts add to the unlikelihood of obtaining beers of exactly the same physical and organoleptic properties from barley and sorghum malts (Owuama, 1999). Thus, it is expected that sorghum beer of a slightly different character eg. in colour, flavour and taste will be produced. Producing beer with 100% sorghum immensely benefits coeliac disease sufferers who are allergic to gluten, which is present in barley beer (Tanner et al., 2013). Currently, wholly sorghum beer is commercially available and does have great appeal to coeliac disease patients. Hopefully, sorghum beer will attract a wider range of consumers in the near future, particularly among the younger generation.

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I designed, searched literature and prepared the manuscript for submission.

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Immune Response after Three Doses of Hepatitis B Vaccine among Children below Five Years of Age in Mwanza, Tanzania

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Abstract- Background: Hepatitis B virus (HBV) infections is moderately endemic in many countries in the sub-Saharan Africa including Tanzania. Immunization of children below five years of age has been found to be an effective strategy in controlling infectious diseases. However, the data regarding immune responses following vaccination are very limited in low-income countries. Here, we report the sero-conversion among children below five years of age after three doses of HBV vaccine in Mwanza, Tanzania.

Methodology: A cross-sectional study involving children below five years of age was conducted at Makongoro Reproductive and Child Health (RCH) clinic between May and June 2017. Socio-demographic data were collected, and vaccination status was confirmed from reproductive and child health (RCH) cards. Serum HBV surface antibodies (anti-HBs) were quantified using enzyme immunoassay (Enzygnost Anti-HBs II). Data were analysed by using STATA version 13 software.

Keywords: hepatitis B, children, immune response, seroconversion, mwanza, Tanzania.

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Results: A total of 300 children were enrolled with the median age of 15 (Interquartile range [IQR]: 9-22.5) months. The median interval from last dose to the time of evaluation was 10(IQR: 5-18) months with all children assessed ≥8weeks post-vaccination. Out of 300 children, 265(88.3%, 95% Confidence interval [CI]:84-91) had ≥ 10IU/L of anti -HBs indicating sero-conversion. The median titres among those who seroconverted was 66(IQR: 26-134) IU/L. The median age at the time of evaluation of those who seroconverted was significantly lower than those who were not seroconverted [14(IQR: 9-21) vs. 18(IQR: 11-26, P=0.03]. The titres were found to decrease significantly by 0.84 and 0.98 IU/L with a unit increase in interval from last dose and a unit increase in age, respectively.

Conclusion: There is high seroconversion after three doses of HBV vaccine among children in Tanzania which is associated with young age. Further studies to evaluate immunogenicity of HBV vaccine in different age groups are recommended in resource limited countries to provide data that regarding booster dose recommendation. In addition, there is a need to evaluate immunization efficiency by determining immunogenicity of one of the vaccine component in the package as a proxy indicator.

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I. Backgrounds

Hepatitis B virus (HBV) infection is one of the most common diseases across the globe with one third of the population estimated to be infected[1]. About 5% of total world population are chronic carriers and nearly a quarter of these carriers develop liver cirrhosis and hepatocellular carcinoma[2] with about one million deaths being reported annually [3]. Therapeutic options for treating HBV chronic infections are difficult to implement and are not yet fully effective in many settings particularly in resource limited countries. Vaccination remain to be an effective measure to prevent HBV infections. Effective vaccination has been found to reduce HBV infections, therefore reducing the risk of transmission to the susceptible contacts[4]. In the intermediate and high endemic regions, individuals are at high risk of acquiring HBV infection if vaccination is not implemented [1]. The World Health Organization (WHO) recommends that HBV vaccination should be part of national immunization programs for countries with HBV carrier prevalence of 8% or greater, to reach a goal of reducing a proportion of chronic carriers and complications associated with HBV infections [5, 6].

In Tanzania the inclusion of HBV vaccine in childhood immunization program was first implemented in 2013 and the vaccine is administered 0.5ml intramuscular on fatty tissue over anterolateral thigh muscle at weeks 6, 10 and 14 respectively after birth in combination with other 4 vaccines in the package (pertussis, diphtheriae, tetanus and *Haemophilus influenza* type B). It is estimated that this standard schedule of immunization should produce about 95% seroprotection [6]. Despite the reported high seroconversion following HBV vaccination in other countries, there are variations in these proportions among different geographical areas with different endemicity status[7]. A previous study[8] in Dar es Salaam among children below five years of age reported sero-conversion of 69%.

Different factors including storage conditions, different forms of immunosuppression, genetic makeup etc. have been implicated to affect the immune response to HBV vaccination[9, 10]. In the countries like Tanzania, where there is no routine assessment of immune response which will lead to additional dose for non-responders, there is a need of data to evaluate DPT-HBV programme after 6 years of its implementation. In addition, there is limited data on the efficiency of childhood immunization particularly in vaccines which are given in combinations in Tanzania. Some previous studies evaluated efficiency of childhood immunization by using other components such as diphtheriae and tetanus toxoid (TT) vaccine [11-13] while others used pertussis component. In a view of that, the study was designed to assess the immunogenicity of HBV vaccine among children who completed three doses, the information that may be useful in controlling vaccine preventable diseases in Tanzania.

II. Methods

a) Study design, study area and study population

The cross-sectional study was conducted from May to June 2017 among children under five years of age from Makongoro reproductive and child health (RCH) clinic. This facility had no any report of cold chain problems in routine assessment.

b) Sampling and inclusion criteria

The sample size was calculated using Kish Lisle formula using the prevalence of 87%[14]. Children under five years of age who had received three doses of HBV vaccine (Pentavalent Vaccine-DPT-HepB-Hib) were serially enrolled until the sample size was reached. The study included only children who had received three doses of Pentavalent Vaccine (DPT-HepB-Hib) with the last dose given at least 8 weeks ago. To avoid non-responders due to chronic HBV infection, all children who were HBsAg positive were excluded from the study.

c) Laboratory procedures

About 3ml of venous blood was aseptically collected using plain vacutainer tubes (BD, Kenya, and Nairobi) and transported to BMC accredited laboratory for processing. The anti-HBs titres were quantified using enzyme immunoassay (SIEMENS, Enzygnost® Anti-HBs II, and Germany) following manufacturer's instructions to detect the presence of specific anti-HBs. The presence of anti-HBs greater than 10IU/L was defined as presence of protective antibodies.

d) Data management and analysis

Data were entered and analysed using a STATA version 13. Continuous variables were summarized as median with inter-quartile range and categorical variables were summarized as proportions. Rank sum-Mann Whitney test was used to compare the median titres, weight, age and interval from the last dose. Using immune response as outcome, multivariate logistic regression analysis was done. However, weight and interval were not included in the model because of their collinearity with age. In all children with titres greater than 10IU/L, regression analysis was done to determine the correlation between age, interval from the last dose and titers. A predictor with a *P* value of <0.05 was considered statistically significant.

III. Results

a) Socio-demographic characteristics of the enrolled children

A total of 300 children under five years of age who received three doses of HBV vaccine were enrolled. There was almost equal distribution between females (49%) and males (51%). The median age of enrolled children was 15 (Interquartile range [IQR]: 9-22.5) months. The median interval from last dose to the time of evaluation was 10(IQR: 5-18) months with all children assessed ≥8weeks post -vaccination. The median duration for breastfeeding was 12(IQR:9-15.5) months (Table1). All children had no co-morbidities.

Table 1: Characteristics of enrolled children and seroconversion

Variable	Univariate analysis			Multivariate	
	Positive	Negative	P-value	OR[95%CI]	P-value
Age(months)	14(IQR:9-21)	18(IQR:11-36)	**0.030**	0.96(0.94-0.99)	**0.005**
Breastfeeding duration (months)	12(IQR:9-16)	12(9-14)	0.766	1.29(0.59-2.79)	0.514
Interval from the last dose(months)	10(IQR:5-17)	13(IQR:5-31)	0.103		
Weight(kgs)	9.5(IQR:8.4-11.2)	10.4(IQR:9-13.5)	**0.014**		
Sex					
Female	127(86.4)	20(13.6)			
Male	138(90.2)	15(9.8)	0.305	1.39(0.67-2.87)	0.370

b) Seroconversion and associated factors

Out 300 children, 265(88.3%, 95% Confidence interval [CI]:84-91) had≥ 10IU/L anti -HBs indicating sero-conversion. The median titres among those who seroconverted was 66(IQR: 26-134) IU/L. The median age at the time of evaluation of those who seroconverted was significantly lower than those who were not seroconverted [14(IQR: 9-21) vs. 18(IQR: 11-26, P=0.03] (Figure1).

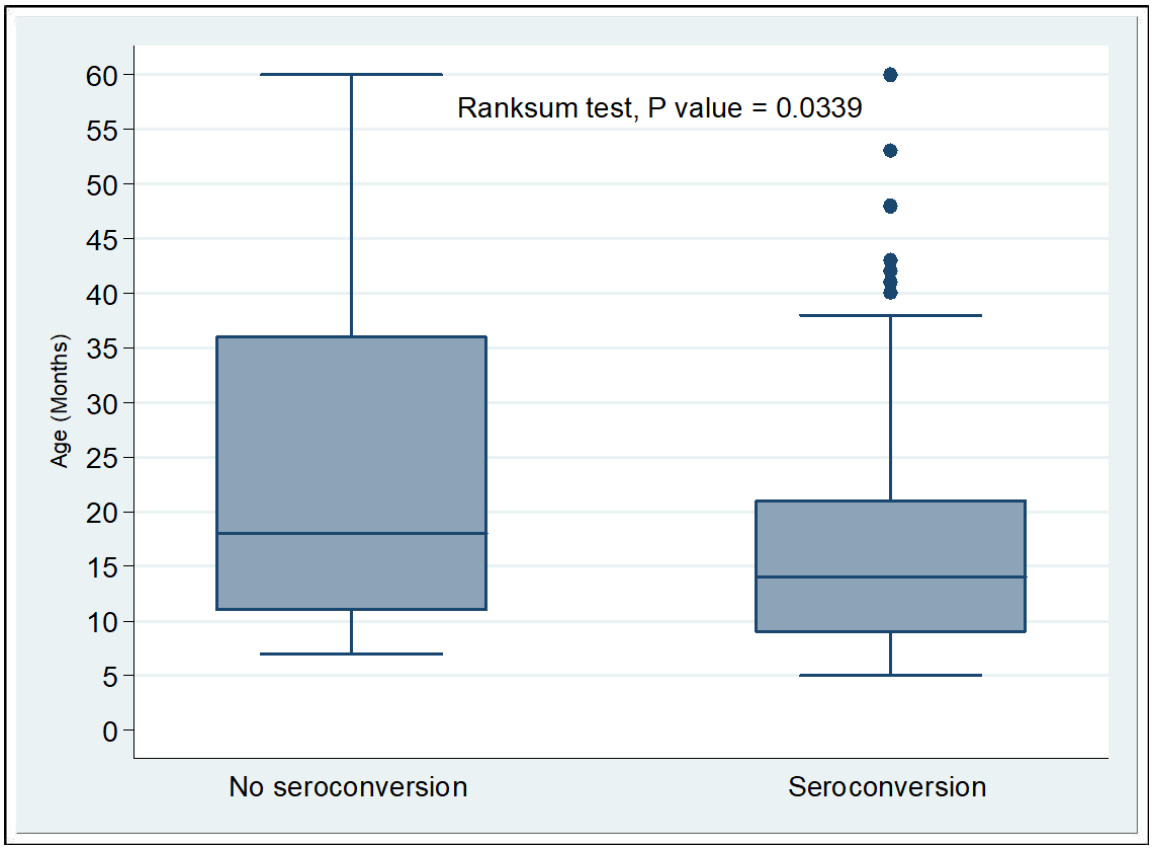

Figure 1: Box plot showing median age of those with seroconversion and those with no seroconversion

By multivariate logistic regression analysis decrease in age was independently associated with seroconversion (OR: 0.96(95%CI: 0.94-0.99, *P*=.005). The titres were found to decrease significantly as the age increases (Coef= -0.96, 95%CI -1.700 - -0.274, *P*=.007) (Figure2).

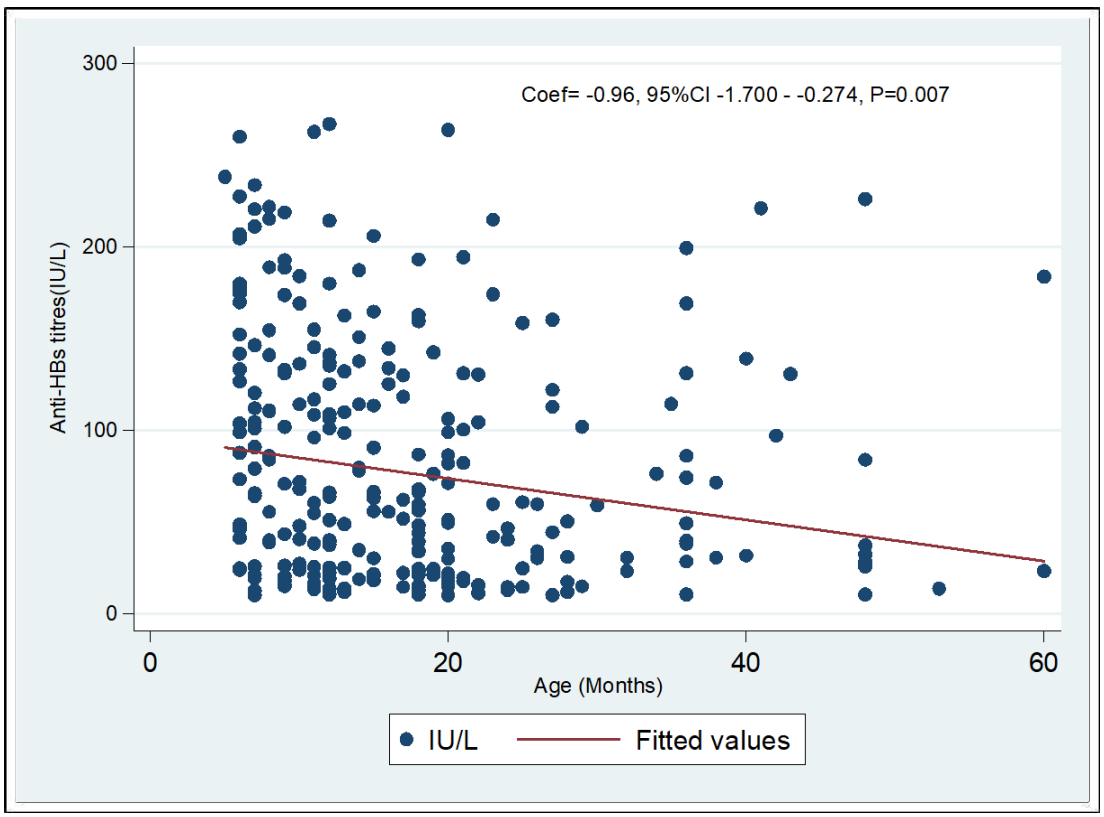

Figure 2: Scatter diagram with fitted line showing correlation between age and anti-HBs titres

In addition, the titers were also found to decrease as the interval from the last dose increases (Coef= -0.84, 95%CI, -1.591- -0.106, *P*= .025) (Figure3).

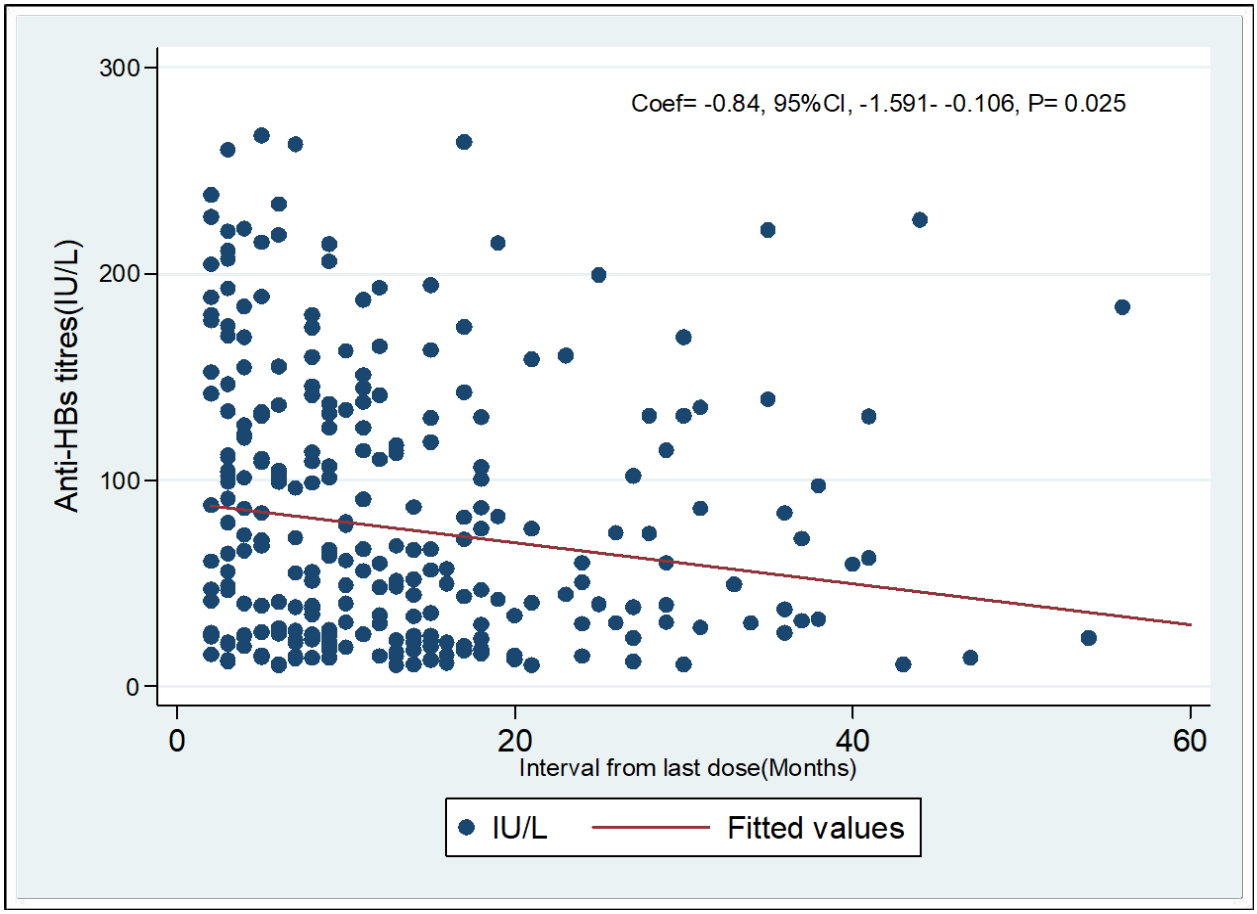

Figure 3: Scatter diagram with fitted line showing correlation between interval from last dose and anti-HBs titres

IV. Discussion

One of the key aspects in vaccination programs in resource constrained countries is to ensure potency of the vaccine by maintaining the cold chain. Therefore, there is a need for regular studies to assess immunogenicity of vaccines especially those which are given in combination to provide a proxy indicator for the efficiency of other vaccines in the package. In Tanzania, HBV vaccine is given in combination with *Corynebacterium diphtheriae*, *Bordetella pertussis* (whooping cough), *Clostridium tetani* (tetanus) and *Haemophilus influenza* type B (influenza). However, there is paucity of data on immunogenicity of these vaccines.

To the best our knowledge, this is the first study to assess immune response after HVB vaccine among children in Mwanza, Tanzania. In the present study about 90% of children seroconverted after three doses of HBV vaccine. The observed high seroconversion and sustained high HBV vaccination coverage of 92.5% [15] reported in Mwanza will eventually reduce the transmission of HBV in future. In addition, this information can be used as a proxy indicator for efficiency of other vaccines given in combination with HBV vaccine in Tanzania. The reported seroconversion rate in the current study is consistent with the previous reports which documented the seroconversion of 87%, 81.5%, 94.1 and 96.7% [14, 16-19]. In the contrary, the reported seroconversion in the current study is higher than reported previously in Dar es Salaam and other endemic areas[8, 20]. Variations in seroconversion might be attributable to the type of vaccine used in terms of synthesis and preparations etc; in the current study the vaccine used was Pentavalent Vaccine (DPT-HepB-Hib) which might be different from other studies where monovalent HBV was used [14, 16]. In addition, amount of antigen delivered, genetic variation among the population involved, vaccination coverage, endemicity status, faults in vaccine cold chain, methods used to evaluate antibody titers etc. might contribute to the observed discrepancies [21-24]. Moreover, in this study, about 11.7% of children were found to be non-responders after receiving three doses of HBV vaccine which is slightly lower than 14.6% and 15.6% reported in previous studies[25, 26]. The possible explanation could be genetic variability and impaired lymphocyte activation as reported earlier [19, 27-29]. It should be noted that children studied in the current study were healthy with

no co-morbidities. Genetic factors and primary immunodeficiencies could were not ruled.

In the current study, it was observed that, as the age increases by one month, the anti-HBs titers were found to decrease by 0.96 IU/L. It was further observed that, the anti-HBs titers decrease by 0.84IU/L as the interval from the last dose increases by one month. With this trend, by the age of 10 years most of these children would have undetectable levels of anti-HBs titers necessitating the need for considering booster dose to provide long lasting protection. Cohorts with long term follow-ups are recommended in this setting to evaluate the need for a booster dose. This observation is consistent to what was reported earlier [16, 20, 30-32] whereby the anti-HBs titers were found to decrease as the age increases and almost undetectable to a significant proportion of children by the age of 11 years. In the contrary some other studies concluded that, there is no need for booster dose after receiving 3-dose schedule of HBV vaccine since the anti-HBs titers can persist for longer period [33] while another study confirmed that there is long lasting cellular immunity despite decrease anti-HBs levels[34]. This conflicting information could be due to endemicity status in the study areas. Further studies to evaluate the levels of anti-HBs titers and cellular immunity among different age groups are highly recommended in areas with different endemicity status.

Regarding sex, in the current study, there was no significant difference in the levels of anti-HBs titers among female and male children which is similar to the previous reports [14, 25, 35]. This could be explained by the fact that there was almost equal distribution between males and females with equal distribution of factors that could influence seroconversion and level of titres.

Limitations of this study include: Inability to assess other forms of primary immunodeficiencies and genetic conditions which might impair immune response to vaccines and contributes to a significant proportion of non-responders and failure to give birth dose as it is not included in Tanzania Immunisation Vaccination and Development Program.

V. Conclusion

There is high seroconversion after three doses of HBV vaccine among children in Mwanza city which is associated with young age. Further studies to evaluate the level of protective antibodies at different age groups are recommended across the country and other resource constrained countries. This necessary especially in deciding the issue of dose at birth and booster dose in ration to HBV vaccination. High seroconversion of HBV vaccine signifies the effectiveness of other childhood vaccines in Tanzania.

List of abbreviation

Anti-HBs: Antibodies against hepatitis B surface antigen
CUHAS: Catholic University of Health and Allied Sciences
CI: Confidence interval
ELISA: Enzyme-linked immunosorbent assay
HBV: Hepatitis B Virus
HBs: Hebatitis B surface antitigen
IU: International Unit
IQR: Interquartile range
OR: Odd ratio
TT: Tetanus toxoid

Declaration

Ethical approval and consent to participate

The ethical clearance to carry out the study was approved by the joint Catholic University of Health and Allied Sciences/Bugando Medical Centre (CUHAS-BMC) research ethics and review committee (CREC) with ethical clearance number CREC/118/2016. All the parents/guardians were asked to sign informed consent on behalf of their children prior enrollment to the study.

Consent for publication

Not applicable

Availability of data and material

All data were included in this manuscript. The raw data is available upon request to the Director of research and Innovation of the Catholic University of Health and allied Sciences.

Competing of interests

No conflict of interest to declare.

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Authors' contributions

DRM, SK, RR, EK, TM, MMM and SEM participated in the design of the study. EM and DRM participated in the collection of specimens and data. MMM, SEM and EM performed serological tests. SEM and MMM analyzed and interpreted the data. DRM wrote the first draft of the manuscript. SEM and MMM provided a critical review of the manuscript. All authors read and approved the final version of the manuscript.

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Ethnobotanical Survey and Antibacterial Activity of African Plants used Against Diarrhea

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Abstract- *Context:* In Africa, medicinal plants are usually used to treat diarrhea but few data are available concerning their identification and their antidiarrheal activities.

Aims: This study aims to record some antidiarrhoea plants in the Medoneu locality (Gabon), describe their modalities of use and assess the chemical composition and antibacterial activities of four most commonly used plants.

Methods and Material: Plants and their modalities of use were recorded through ethnobotanical survey. Four commonly used plants were harvested; their extracts and the essential oils (EO) were prepared from the leaves by maceration and hydro-distillation. The chemical compositions of EO were determined by Gas Chromatography while, the antibacterial activities of extract and EO were assessed using both agar diffusion and micro-dilution methods.

Keywords: *diarrheal infections; african medicine, plants extracts; essential oils, antibacterial activity.*

GJMR-C Classification: *NLMC Code: QW 60*

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Nyegue Maximilienne Ascension [α], Voundi Olugu Stève Henri [σ], Lepengue Nicaise Alexis [ρ], Ndong Aveme Rodrigue [ω] & Etoa François-Xavier [¥]

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Results: The most represented families of plant used to treat diarrhea were *Euphorbiaceae.* Leaves and barks were the most used organs. Decoction and maceration appeared as the most common preparation methods and oral administration the most used route of administration. The EO of *Psidium guajava, Lantana camara* and *Ageratum conyzoïdes* were mainly composed of (E) -β-caryophyllene (26.5%), (E) -nerolidol (26.4%) and germacrene D (41.6%) respectively. The EO inhibited the growth of bacterial species with the smallest MIC of 6.25 obtained with *P. guajava* EO. Plant extracts were more active compared to EO, the smallest MICs of 0.19 to 6.25 mg / mL were obtained with *P. guajava* extracts

Conclusions: These results explain the use of different plants in Africa medicinal to treat diarrheal infections and present four of them as potential source of antimicrobial compounds.

Keywords: diarrheal infections; african medicine, plants extracts; essential oils, antibacterial activity.

Key Messages: A great diversity of African medicinal plants is used in the treatment of diarrhea. Some of these plants included in the study are *Psidium guajava, Lantana camara, S. acuta* and *Ageratum conyzoïdes* have shown to have antimicrobial activities on the growth of some bacterial species causing diarrhea.

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I. Introduction

Diarrheal infections are generally caused by bacteria, fungi or some viruses leading to digestive tract dysfunction. Although these infections are sometimes transient, acute forms can quickly get worse and lead to death or other severe adverse effects in some vulnerable people such as children under five, HIV/AIDS and individuals with an aging immunity [1]. To date, diarrhea is one of the most common and widespread diseases worldwide, particularly in African marginalized communities where it causes millions of deaths yearly [2]. Diarrhea is more deadly than malaria and tuberculosis. It is responsible of about 1.5 million of deaths in children each year worldwide. It is the third leading cause of death in low and middle income countries and sub-Saharan African populations are the most at risk [3]. In Gabon and Cameroon, diarrhea is the third leading cause of morbidity with a prevalence of 13 to 19 % [4].

Treatment of diarrhea involves oral rehydration as the first-line therapy (WHO and United Nations Children's Fund (UNICEF) but, in case of severe infections antibiotics are conventionally used. However, there is an upsurge of antibiotic resistance due to the loss of their efficacy resulting from inappropriate use or the poor management of infections. Besides, the high cost of antibiotics and their unavailability justify the resortment of those populations to traditional medicines [5, 6]. Use of traditional medicines is a fundamental component of African traditional health care system. About 80% of African populations rely on medicinal plants for various ailments and diarrheal infections particularly because appropriate health centers are often very far [7], [8], [9]. However, there is paucity of detailed scientific information on the composition and bactericidal effects of those plants which can constitute an alternative and or complementary method to the use of antibiotics.

Ageratum conyzoïdes, *Lantana camara*, *Psidium guajava* and *Sida acuta* are some of the plants found in Gabon and Cameroon flora. They are used in traditional pharmacopeia to treat various diseases [5, 10]. However, there are very few scientific research data on their antibacterial potential. Hence, the present work was carried out to study the growth inhibition effects of crude extracts and essential oils of *Ageratum conyzoïdes*,

Lantana camara, *Psidium guajava* and *Sida acuta* on five diarrhea-causing bacteria species as well as their phytochemical composition after an ethnobotanical survey which shows that they are traditionally used in Gabon to treat diarrheal diseases.

II. Subjects and Methods

a) Ethnobotany survey, harvest and identification of plants

The first step of the study was to carry out the ethnobotanical survey, to compile the data regarding the medicinal plants use against diarrheal infections, the mode of preparation and the route of administration. This survey was carried out in Gabon, in the Region of Woleu-Ntem, city of Medouneu with coordinates 0 ° 57'00 'North and 10 ° 47'00' 'East. The survey was conducted by administration of verbal questionnaire to 100 traditional medicinal healers locally called "Ngueguan" which means holder of ancestral medicinal knowledge. The purpose of this survey was to identify the most used plants in the treatment of diarrhea, to report the used plant part, the mode of preparation and the route of administration. The most cited plant were selected, photographed and botanically identified at the Gabonese National Herbarium at Libreville.

The four most cited plants namely *Ageratum conyzoïdes*, *Lantana camara*, *Psidiumguajava* and *Sidaacuta*were harvested from the botanical reserves of the University of Yaounde I with coordinates 3 ° 51'25 'North and 11 ° 30'05' East. Indeed, Cameroon and Gabon are two bordering countries with very close flora and culture, which explains the similarities in the treatment of diseases in their traditional pharmacopoeia. All plant harvests were made during November and December 2015, early in the morning (before 6: am) and involved the removal of leaves that were used for this study. The plant samples used for the extraction of the non-volatile compounds were dried at room temperature for two weeks and ground to obtain a powder.

b) Bacterial strains

Five bacterial species were used for antimicrobial tests. *Escherichia coli* ATCC, *Staphylococcus aureus* ATCC 13565 and *Bacillus cereus* T are referenced strains coming respectively from American Type Culture Collection (for the first two) and the Institute of Food Research in Reading. These last, are responsible for enterotoxigenic diarrhea. To these three species were added two clinical isolates including *Salmonella enteritidis* and *Shigella spp*., two bacterial species frequently responsible for entero-invasive diarrhea. These bacterial strains were kept at the Laboratory of Microbiology of the University of Yaounde I where antibacterial tests were carried out.

c) Extraction of essential oils

Extraction of the essential oils was carried out by hydrodistillation using a Clevenger type apparatus as described by Nyegue [11]. 300 g of leaves of each plant material were submitted to hydro-distillation using the Clevenger-type apparatus for 6 hours. The EO were separated from the mixture water-EO by decantation and then introduced into a dark bottle. After weighing, the obtained EO was dried over anhydrous sodium sulfate and stored at +4°C before used [12]. The extraction yields of EO expressed as a percentage (%) were calculated according to the formula Yield (%) = Mass of essential oil / Mass of raw plant material X 100.

d) Preparations of crude extracts

All the extractions were carried out by maceration using for each plant, three different solvents namely water, ethanol and the hydro-ethanol mixture (V / V). For this, 300 g of powder of leaves of each plant were immersed separately in 1500 ml of water (aqueous extract), pure ethanol (ethanolic extract) and ethanol-water mixture (V / V) (hydro ethanolic extract). The maceration was carried out for 48 hours with regular shake. At the end of this time, the solutions were filtered with hydrophilic cotton followed by Whatman paper N° 3. The filtrates collected from ethanolic extracts were evaporated at 70 ° C using a rotavapor, while the filtrates from aqueous and hydroethanolic extract were submitted to lyophilisation. The extracts obtained were kept at 4 ° C before used. The yields of the extractions were calculated according to the formula: Yield (%) = Mass of extract / Mass of raw plant material X 100 [13]

e) GC/FID and GC/MS analysis of the essential oil

The chemical composition of the EO was realized by GC/FID and GC/MS method as described by Kemegne et al. [14] GC/FID analysis was carried out using a TRACE 1300 Thermo scientific instrument equipped with two fused silica capillary columns DB-5 (30m*0.25mm*0.25μm) and DB-Wax (30m*0.25mm*0.25μm), programmed from 60-220°C at 3°C/min with a final hold time of 17 min, carrier gas Ultra High Purity N_2 at a split flow of 10 ml/min and purge flow of 5 ml/min, injector at 220°C.

GC/MS analyses was carried out using a Agilent 5977 MSD serie (Agilent technologies) apparatus equipped with two silica capillary columns HP-5MS (30m*250μm*0.25μm), HP-INNOWAX (30m*250μm*0.25μm) and interfaced with a single quatrupole detector. Column temperature 60-240°C at 3°C/min; injector temperature 240°C; carrier gas, He, at a flow rate of 0.7 ml/min; injector type split 20:1; the spectrometer was operated at 70.0 eV; mass range 33-400 and scan acquisition type.

Injections of authentic reference compounds, determination of their linear retention indices relative to the retention times of a series of n-alkanes as well as published mass spectra (9-21) and retention indices

were used as basis for the identification of the constituents, which were quantified as area percentage of total volatiles from electronic integration. Results obtained on HP-5 column permitted to the identification of components in comparison to some available data base (NIST14, NIST98, FFNSC 2.L, ESSENCES L.) and to literature data. Results obtained on Carbowax permitted to confirm one obtained on HP-5 column.

f) Phytochemical analyses

To identify the major phytochemical families of compounds present in the crude extracts, qualitative phytochemical analyses were carried out according to qualitative methods described by Odebiyi and Sofowora [15] and Harbone [16].

i. *Test of alkaloid:* 50 mg of the sample of each plant extract were dissolved in 10 mL of H_2SO_4 2%. The mixture was homogenized and boiled for 2 min before being filtered. 1 mL of the filtrate was added to five drops of Mayer's reagent. Formation of turbidity or precipitation was taken as evidence for the presence of alkaloids in the extract.

ii. *Test of phenols and polyphenols:* 50 mg of the sample of each plant extract were dissolved in 2 ml of water and this solution was added to 3 ml of fresh solution of $FeCl_2$ 5%. After homogenization, five drops of potassium ferricyanide 1.00% were added. Formation of a green or blue precipitate was considered as evidence of the presence of phenol or polyphenols respectively.

iii. *Test of flavonoids:* 50 mg of each extract were dissolved in 5 mL of methanol. To this solution were added some magnesium chips and drops of concentrated HCl. The presence of flavonoids was revealed by the appearance of orange or purple color.

iv. *Test oftriterpenes and steroids:* A vegetable preparation was obtained by dissolving 50 mg of extract in 20 ml of methylene chloride. To this solution were successively added 4 drops of acetic anhydride and sulfuric acid. The presence of triterpenes was revealed by the appearance of the purplish red color whereas the greenish blue color was considered as characteristic of steroids.

v. *Test of saponin:* 25 mg of plant extracts were solubilized in 15 ml of distilled water and the solution boiled in a water bath for 5 minutes. After cooling, the solution was mixed. The presence of persistent foam more than 1 cm thick for at least one minute indicates the presence of saponins

vi. *Test of Anthocyanin:* 50 mg of plant extract were dissolved in 15 ml of HCl 1% and the mixture was boiled for 5 minutes. The change of the color from orange-red to orange-blue reveals the presence of anthocyanins.

vii. *Test of Tannins:* To 15 ml of an alcoholic or aqueous solution of plant powder, were added a few drops of iron chloride. A color change reflects the presence of tannins. The dark coloration was characteristic of gallic tannins and the green color revealed the presence of catechism tannins

g) Evaluation of antibacterial effects

The antibacterial assay consisted of determining the sensitivity of the bacteria to the extracts and subsequently evaluating the inhibition parameters (MIC and CMB). The sensitivity of bacteria was assessed by the agar diffusion method using disks for the EO and from the wells for non-volatile extracts [17]. The MIC and MBC (Minimum Inhibitory Concentration and Minimal Bactericidal Concentration) were then determined by the micro-dilution method as described by Ateufack et al. [18] recommended in the standard protocol of CLSI-M-A9 [19].

i. *Agar diffusion method (Antibiogram)*

The test was performed by swabbing a suspension of the overnight tested microorganism load of 0.5 McFarland scale (corresponding to approximately 1.5×10^6 cells/mL standardized with a spectrophotometer) on the Muller Hinton Agar. The solutions of each extract were prepared at 100 mg / mL, using $Tween_{40}$ 5% for the EO and sterile distilled water for the nonvolatile extracts. Disks impregnated with 15 µL of solution of each EO at 1 mg / mL or gentamicin at 1 mg / mL (reference antibiotic) were then deposited on the inoculated media. Concerning the extracts, wells of 6 mm of diameter were punched on the agar medium and each well was filled with 50 µL of extract solution. After 30 minutes of pre-diffusion at room temperature, the plates were incubated at 37 ° C for 24 hours, the diameters of the microbial growth inhibition zones were measured and the averages calculated from the repetitions of three tests.

ii. *Determination of Minimal Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)*

MIC and MBC were determined by the culture broth microdilution method according to the standard protocol of CLSI-M-A9 (2012) using 96-well microplates. All the tests were performed in three replicates. In each well of the microplate, were first introduced 100 µL of Muller Hinton broth (supplemented with $Tween_{40}$ at 5% for th EO). Subsequently, 100 µL of 200 mg / mL solutions of each extract or EO were added to the first wells. The dilutions of the extracts or EO were done according to a geometric progression reason 2 until the concentration of 0.048 mg / mL. 100 µL of inoculum prepared with overnight tested microorganism were then inoculated into each well to obtain a final load of 10^6 cells / mL and the whole plate incubated at 37°C. After 24 hours of incubation, the bacterial growth was revealed by addition of 40 µL of a solution of 2,3,5-

Triphenyltetrazolium Chloride (TTC) 0.01% in each well. This was followed with 30 minutes of incubation and the appearance of a red color indicates the presence of living bacteria in the medium. The smallest concentration of extracts or EO not showing the red color represents the MIC.

The MBC were determined by inoculation in new microplates, 50 µL of inoculum (with concentrations greater than or equal to the MIC) in 150 µL of nutrient broth contained in new wells. The plates were then incubated for 48 hours at 37 ° C, before being revealed with TTC as previously described. After 30 min of incubation, all concentrations not showing red color were considered as bactericidal. The smallest of these has been noted as MBC.

The MBC / MIC ratio were calculated to determine the bactericidal effect (MBC / MIC <4) or bacteriostatic effect (MBC/ MIC ≥ 4) of the test substances [20].

iii. *Statistical analysis*

Inhibition diameter values were statistically analyzed by ANOVA at the 5% probability level ($p < 0.05$) using GraphPad prism 5.0 software. The Excel spreadsheet was used to calculate means and standard deviations.

III. Results

a) Ethnobotanical survey

The ethnobotanical survey was conducted in the locality of Medouneu (Gabon) in order to identify the commonly used plants for the treatment of diarrhea. The part of plant used, the mode of preparation and the route of administration were the main points of the survey. The results summarized in Table 1 present 24 identified main plant families namely *Anacardiaceae*, *Annonaceae*, *Apocynaceae*, *Aspleniaceae*, *Asteraceae*, *Bombacaceae*, *Caesalpinaceae*, *Canellaceae*, *Chenopodiaceae*, *Combretaceae*, *Convovulaceae*, *Ebenaceae*, *Euphorbiaceae*, *Fabaceae*, *Hypericaceae*, *Labiaceae*, *Leguminosae*, *Lycopodiaceae*, *Malvaceae*, *Mimosaceae*, *Moraceae*, *Myrtaceae*, *Sterculiaceae* and *Vernaceae*. The most represented family was that of *Euphorbiaceae* with 8 different genera and species (*Acalypha reticulate*, *Alchornea cordifolia*, *Bridelia atroviridis*, *Euphorbia hiria*, *Macaranga* spp., *Hymenocardia acida*, *Jatropha curcas* and *Uapaca densifolia*).The second most encountered family was the *Asteraceae* with 6 species (*Acanthospermum hispidum*, *Ageratum conyzoïdes*, *Apodocephala pauciflora*, *Biden spilosa*, *Vernonia cephalophora* and *Vernonia amygdalina*), followed by *Anacardiaceae* with 5 species (*Anacardium occidentale*, *Lannea acida*, *Lannea microcarpa*, *Mangifera indica* and *Sclerocarya birrea*). *Combretaceae* were also highly represented with 5 species (*Anogeissusleiocarpus*, *Combretum micranthum*, *Combretum nigricans*, *Guiera senegalensis* and *Terminalia avicenioides*). The other plant families were represented by one or two species generally belonging to different genera, except *Annona muricata* and *Annona senegalensis* both belonging to *Annonaceae* family.

Table 1 reveals a great diversity of plant organs namely barks, leaves, stem, stem bark, fruits, seed, pulp roots and entire plant (in some cases) used by the population in the treatment of diarrhea. However, the type of organs used varied according to the plant but in general the leaves and barks were the most used organs except for *Lannea microcarpa* and *Biden spilosa* where young leaves have been recommended. About the method of preparation, various methods were also recorded although decoction and maceration appeared as the most used methods. Infusion was recommended only in the case of *Apodocephala pauciflora* and *Chenopodium ambrosioides (leaves)* and the entire plant of *Ocimum sp.* Likewise, preparation by expression was mentioned only for the leaves of *Macaranga sp.* In addition, oral administration was the most common route although bath was recommended in the case of stem and leaves of *Merremia pellaia,* barks or stems of both *Parkia biglobosa* and *Ficus sycomorus*.

b) Extraction yields

After maceration and hydro distillation, the extraction yield values of different plants sample were recorded in Table 2. It appears that the extraction yields vary from one plant to another and depend on the solvent used. Overall, the lowest yields were obtained with EO, while the highest percentages were obtained with hydro ethanolic extraction. *A. conyzoïdes* leaves showed the highest EO yield of 0.21 ± 0.02%, while *S. acuta* did not have any essential oils. The highest yields were obtained from hydro ethanolic extracts of *A. conyzoïdes* (11.20%), followed by *L. camara* (10.33%) and *P. guajava* (9.74%). The highest extraction yield value for *S. acuta* (3.73%) was obtained with the aqueous extract.

c) Essential oils composition

Table 3 presents the results of the chemical composition of the essential oils (EO) of *P. guajava*, *L. camara* and *A.* conyzoïdes analyzed by Gas Chromatography- FID (GC-FID) and GC coupled to Mass Spectrometry. Analysis of *P. guajava* EO enables to identify 61 compounds representing 99% of the EO. The oil is composed by 8.9% of aliphatic compounds, 6.2% of monoterpenes and 83.9% of sesquiterpenes. *P. guajava* EO was characterized by 8 main components accounting *(E)*-*β*-caryophyllene (26.5%), *β*-bisabolol (8.9%), benzaldehyde (7.8%), *(E)*-nerolidol (7.2%), 1,8-cineole (5.7%), *β*-sesquiphellandrene (4.3%), *α*-humulene (3.9%) andisodaucene (3.7%). *(E)*-*β*-caryophyllene is therefore the main component of the oils.

In the other hand, analysis of *L. camara* EO allowed to identify 55 compounds over 61 representing 99.4% of the total EO. The EO is composed by 23.9% of monoterpenes, 73.8% of sesquiterpenes and 1.6% of diterpenes. It contains7 main components namely *(E)*-nerolidol (26.4%), *(E)*-*β*-caryophyllene (12.7%), sabinene (8.4%), 1,8-cineole (6.9%), *α*-zingiberene (5%), *α*-humulene (4.6%) and *γ*-curcumene (3.8%). Nevertheless, *(E)*-nerolidol (26.4%) and *(E)*-*β*-caryophyllene (12.7%) are the main compounds of the oils.

Chemical analyses of *A. conyzïdes* EO revealed 16 compounds representing 88.6% of the total EO. The class of hydrocarbon monoterpenes with 5.8% of the total EO is poorly represented. On the other hand, germacrene D (41.6%) and β-trans-caryophyllene (24.6%) are the major compounds of EO conferring it an important composition in sesquiterpenes.

d) Phytochemical screening of nonvolatile extracts

The results of the phytochemical screening of non-volatile extracts of each plant are presented in table 4. This results show that the plant extracts studied contain the major families of phytochemicals compounds with antibacterial properties. Anthocyanins, flavonoids and tannins are present in all the extracts. The same observation is made concerning the presence of the alkaloids, except for the ethanolic extracts of *L. camara*and ethanolic and hydro ethanolic extracts of *P. guajava* which are devoid. The polyphenols are present in the aqueous and hydro ethanolic extracts of all the plants, except all the extracts of *L. camara*. Tri terpenes on the other hand are present only in the hydro-ethanolic extracts of all the plants, as well as all the extracts of *L. camara*. However, the presence of phenols is especially noted in ethanolic extracts although their presence is observed in all the extracts of *L. camara*.

e) Antibacterial activities of essential oils and plant extracts

i. *Antibiogram*

Tables 5 and 6 respectively present the values of the inhibition zone diameters of the EO and non-volatile extracts. The analysis of Table 5 reveals that each EO inhibits the growth of at least three out of five bacterial species with overall inhibition diameters between 7.0 ± 0 and 13.6 ± 2.5 mm. The most effective EO is that of *P. guajava*, which exhibited inhibition diameters zone of 12.6 ± 0.5 and 13.6 ± 2.5 mm respectively on the growth of *S. aureus* and *S. enteritidis*. The EO of *L. camara* was also active but only on three bacterial strains (*E. coli, S. enteritidis* and *Shigella* spp.) with inhibition diameters zones between 9 ± 1 and 13 ± 2 mm. The essential oil of *A. conyzoïdes* showed extensive effects on all bacteria, although the observed inhibition zone diameters were < 9.7 ± 0.6 mm. The inhibition diameter zones of the EO of all the plants were 2 to 5 fold lower than those of Gentamicin, the reference antibiotic used.

Tables 6 shows that, the crude extracts inhibit the bacterial growth more significantly compared to the EO ($P < 0.05$) and in diverse manner (extract-dependent effect) with the values of inhibition diameter zones varying between 7 and 16 mm. Concerning *A. conyzoïdes*, only aqueous extract (AE_{AC}) was active, with effects observed on all the bacterial species tested. Values of inhibition diameter zones were ranged from 11.0 ± 1.0 (on *E. coli*) to 12.7 ± 2,1 mm (on *S. aureus*). For *L. camara* the most effects ($P < 0.05$) were obtained with hydro ethanolic extract (HEE_{Lc}) which exhibited effect on all the bacterial species with inhibition diameter zones between 11.0 ± 1.0 (on *B. cereus*) and 15.0 ± 3.5 mm (on *S. enteritidis*). Ethanolic extract (EE_{Lc}) also effective shows antibacterial effect on four out to five bacterial species, with inhibition diameter zones between 9.7 ± 0.6 (on *Shigella* spp.) to 14.3 ± 2.1 mm (on *E. coli*).Aqueous extract of *L. camara* (AE_{Lc}) presented no effect on the growth of all the bacterial species. *P. guajava* presented the same antimicrobial profile as compared to *L. camara* with the best inhibitory potential observed with ethanolic extract (EEpg) and hydro ethalolic extract (HEEpg). The first one exhibited the antibacterial effect on all the bacterial species with inhibition diameter zones ranged from 10.3 ± 0.58 (on *Shigella* spp.) to 11.7 ± 0.6 mm (on *E. coli* and *S. enteritidis*). The other one was active on four out to five bacterial species. However, the latter inhibited more efficiently the growth of *S. enteritidis* with inhibition diameter zone of 16.0 ± 3.6 mm. Regarding *S. acuta*, only hydro ethanolic extract (HEE_{Sa}) was active, with effects observed on all the bacterial species and values of inhibition diameter zones ranging from 8.7 ± 1.5 (on *S. aureus)* to 13.0 ± 2.0 mm (on *S. enteritidis*)

ii. *Determination of inhibition parameters (MIC, MBC and MBC / MIC)*

The results of the growth inhibition parameters (MIC, MBC and MBC/MIC) of the EO, the non-volatile extracts and Gentamicin on bacteria growth are summarized respectively in Tables 7 and 8. Table 7 shows that the MIC of the EO ranges from 6.25 (with EO of *P. guajava*on *Shigella* spp.) to 100 mg / mL (with EO of *A. conyzoïdes* on *S. enteritidis* and *S. aureus*).Overall, the MICs of 25 mg/mL were generally obtained from all the EO although MIC of 12.5 mg / mL was obtained with EO of *L. camara* on *Shigella* spp.

The results presented in Table 8 presented the greater antibacterial effect of the nonvolatile extracts (as compared to the EO) with MICs ranging from 0.19 (with aqueous extract of *P. guajava* on *B.cereus*) to 100 mg / mL (with aqueous extract of *A. conizoïdes* on *B. cereus*). A strong effect was observed with hydroetanolic extract of *L. camara* with MIC values of 0.39 mg/mL on *S. aureus* and 0.78 mg/mL on *B. cereus* and *E. coli.*

Similarly, the same observations were noted with the aqueous extract of *P. guajava* which presented MIC values of 0.19 mg/mL and 0.78 mg/mL on *B. cereus* and *S. aureus* respectively. Likewise, its ethanolic counterpart exhibited the same effect both on *B. cereus* and *S. aureus* with the same MIC value of 0.78 mg/mL. *S. acuta* presented the more effect with MIC value of 0.78 mg/mL with ethanolic extract on *B. cereus* and hydroethanolic extract on *S. enteritidis*. Some good activities can also be noted at MIC value of 1.56 mg/mL as observed with ethanolic extract of *L. camara* on *S. enteritidis*, *shigella spp*. and *S. aureus*; aqueous extract of *L. camara* on *S. aureus*; hydrethanolic extract of *S. acuta* on *B. cereus* and ethanolic extrac of *S. acuta* on *S. aureus*. However, as already observed with the inhibition diameters zones, the activity of the EO and the plant extracts were lower than that of Gentamicin with MIC values between 0.009 and 1.39 mg /mL. The ratio MBC/MIC < 4 is used to determine the bactericidal effect of plant extract on a given strain while, the same ratio greater than 4 is characteristic of a bacteriostatic effect [20]. Thus, the calculation of this ratio showed that most of the EOs from the plants used have a bactericidal effect on all the tested strains, except *B. cereus*, *Sighella* spp. and *S. aureus* where the effects were rather bacteriostatic (Table 7). Non volatile extract have also shown both bactericidal and bacteriostatic activities on the bacterial species tested (Table 8). However, the EO and the extracts showed in more case, bactericidal than bacteriostatic effects on the bacterial species studied.

IV. Discussion

This study allowed the identification of some plants commonly used in Gabon for the treatment of diarrhea, the part of plant used, the mode of preparation and the route of administration. The ethnobotanical survey showed that they are greatly diversify with 52 plant species belonging to 24 main families. This great diversity observed is explained by the geographical and cultural situation of Gabon. Indeed, the country is located in the equatorial zone characterized by the heavy rainfall and the presence of the forest. This forest represents an important medicinal wealth and explains the great biodiversity of plant resources. On the other hand, in Central Africa the natives possess a good knowledge of their natural environment. This knowledge is integrated in important activities of the cultural life, and applied during the rites of initiations, religious and even of cure.

In this study, the most represented families among the cited plants are *Euphorbiaceae*, *Fabaceae* and *Asteraceae*. These results are in accordance with those of Njoroge and Kibunga [21] and Njume and Goduka [22]. These authors also respectively mentioned *Asteraceae*, *Euphorbiaceae* and *Fabaceae* as the most families of plants used in the treatment of diarrhea in other African localities. The leaves and bark were the most part of plant organs used, with various methods of preparation, dominated by decoction and maceration. Oral administration as a drink was the major route of administration. This could be explained by the fact that leaves and bark are perennial parts of the plant and decoction and maceration usually allow the extraction of the majority of plant metabolites. These observations are in the accordance with other studies regarding the use of herbal medicine in the treatments of diarrheal infections[21, 22]. In addition the oral route could be the most used route of administration because it allows bringing the drug directly in the digestive tract facilitating its antidiarrheal effect as compared to the other modes of administration.

Four commonly used plants namely *Ageratum conyzoïdes*, *Lantana camara*, *Psidium guajava* and *Sida acuta* were further investigated to determine their chemical compositions and their effects on the inhibition of the growth of some bacteria responsible of diarrhea. After sample collection and extraction, the yields obtained varied according to the type of extract, plant species and the solvent of extraction. This variability is related to the chemical composition of the plant. Overall, the lowest yields are obtained with EO, while the highest percentages were obtained with hydro ethanolic extraction. This is explained by the fact that the EO are mainly composed by terpenic compounds generally represented at a low percentage in the plant composition. The highest yields obtained from hydro ethanolic extracts is due to the capacity of the solvent to extract both polar and nonpolar compounds as compared to water and ethanol which permit to extract only polar or more non polar compounds respectively.

Phytochemical screening of extracts revealed that the phytochemical components are unequally distributed according to the extract and the plant species. This variability is due to the influence of two parameters, namely the molecular composition of the plant species and the affinity of the solvent with the molecules present in the plant. Indeed, there are differences in solubilization capacity and extraction of solvents with respect to phytomolecules. It has been reported that during liquid-liquid extraction, bioactive substances are distributed among solvents according to their polarity and solubility [23].

Regarding the antibacterial tests, most of the bacterial strains tested were sensitive to the EO and crude extracts. The sensitivity of bacterial species is explained by the presence of families of phytochemicals compounds with antibacterial properties found in the different EO and plant extracts. In fact, concerning the EO, β-caryophyllene presents in all the EO (26.5% in *P. guajava* EO, 12.7% in *L. camara* and 24.6% in *A. conyzïdes*) is sesquiterpene with broad-spectrum antibacterial effects due to its hydrophobicity and ability

to cause structural and functional damage to the cell membrane [24]. Thus, many plant sources of β-caryophyllene are used for several purposes, including antimicrobial activity [25]. Xiong et al [26]. showed wide-spectrum activity of β-caryophyllene against Gram-positive bacteria including *S. aureus*(including methicillin-resistant), *S. epidermidis*, *S. auricularis*, *M. caseolyticus*, *E. faecium* and *E. faecalis* with MIC values ranging from 0.032 to 0.256 mg/mL. Also, Nerolidol (3,7,11-trimethyl-1,6,10-dodecatrien-3-ol) present in the EO of *Lantana camara* (26.4 %) and *Psidium guajava* (7.2%), is a sesquiterpene alcohol present in various plants with a floral odor. The usage of nerolidol is widespread across different industries including cosmetics and food (permitted by U.S. Food and Drug Administration as a food flavoring agent). Nerolidol already showed to possess antibacterial effect due to the disruption of cell membranes. The observed effects could be due to the presence of the long aliphatic chain in chemical structure of nerolidol [27]. The antimicrobial activities of Nerolidol on the growth of the pathogens *Escherichia coli* O157, *Clostridium difficile*, *Clostridium perfringens*, *Salmonella typhimurium* and *Salmonella enteritidis* were already highlighted [28].

Concerning nonvolatile extracts, the broad antimicrobial activity observed can be attributed to the presence of various bio-actives components such as tannins, polyphenols, alkaloids, flavonoids, steroids and saponins found in this extract. The variation observed with the diameters of inhibition zone of the bacteria tested can be attributed either to the difference of the bioactive molecules present in each extract or to their mechanism of action on Gram-positive and Gram-negative bacteria. The mechanism of action of the polyphenols, tannins and alkaloids on Gram-positive and Gram-negative bacteria was demonstrated [29].

The species *L. camara* and *P. guajava* showed strong activity on the strains tested, unlike some previous studies which showed little or no activity with the leaf extracts of *P. guajava* on *S. aureus*, *S. enteridis* and *E. coli* [30].This can be explained by the variation in concentration of active biomolecules due to the plant's harvesting locations (Cameroon) as compared to that used by these researchers. Ecosystem parameters play an important role in the plant phytochemistry responsible for antimicrobial activity in the plant [31].

V. Conclusion

This study reveals a great diversity of the plant used to treat diarrhea in the locality of Medouneu (Gabonese locality) with *Euphorbiaceae*, *Fabaceae* and *Asteraceae* as the most represented families. The leaves and bark are the most plant organs used, with various methods of preparation, dominated by decoction or maceration and oral administration was the most used route of administration. Further studies on some of listed plants namely *Ageratum conyzoïdes*, *Lantana camara*, *Psidium guajava* and *Sida acuta* show the broad antimicrobial activity of the EO and plant extracts on the inhibition of the growth of bacteria responsible of diarrhea. These results explain the use of those plants to treat diarrheal infections and show a potential source of antimicrobial compounds.

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Table 1: List of medicinal plants and their used as recorded by ethno botanical survey

Plant families	Species	Part used	préparation	Administration
Anacardiaceae	*Anacardium occidentale*	barks and leaves	decoction	drink
	Lannea acida	aerial part of plant	decoction	drink
	Lannea microcarpa	young leaves	maceration	drink after filtration
	Mangifera indica	barks or leaves	decoction / maceration	drink
		barks or stem	decoction	bath
	Sclerocarya birrea	barks	decoction	drink
Annonaceae	*Annona muricata*	leaves	decoction	drink
	Annona senegalensis	roots	decoction /maceration	drink
Apocynaceae	*Alstonia congensis*	stem barks	maceration	drink
	Mascarenhasia arborescens	leaves	decoction	drink
Aspleniaceae	*Asplenium monanthes*	fresh fruits	maceration	anal administration
Asteraceae	*Acanthospermum hispidum*	leaves and stem	decoction	drink
	Ageratum conyzoïdes	leaves	decoction	drink
	Apodocephala pauciflora	leaves	decoction / infusion	drink
	Biden spilosa	young leaves	mastication	oral administration
	Vernonia cephalophora	leaves	decoction	drink
	Vernonia amygdalina	leaves	maceration	drink
Bombacaceae	*Adansonia digitata*	Pulp or leaves	maceration or infusion	drink
	Ceiba pentandra	bark	decoction	drink
Caesalpinaceae	*Piliostigma reticulatum*	stem bark	decoction	drink
Canellaceae	*Pentadiplandra brazzeana*	roots	decoction	bath
	Cinnamosma fragrans	bark and stem	decoction	drink
Chenopodiaceae	*Chenopodium ambrosioides*	entire plant/ crush leaves	Infusion / maceration	bath
Combretaceae	*Anogeissus leiocarpus*	leaves	décoction	drink
	Combretum micranthum	leaves	décoction	
	Combretum nigricans	roots	décoction	
	Guiera senegalensis	fruit	décoction	
	Terminalia avicenioides	roots	décoction	
Convovulaceae	*Merremia pellaia*	stem and leaves	decoction	bath
Ebenaceae	*Diospyros mespiliformis*	fruits	maceration	drink
Euphorbiaceae	*Acalypha reticulata*	stem or leaves	decoction	
	Alchornea cordifolia	leaves	maceration	
	Bridelia atroviridis	leaves	decoction	
	Euphorbia hiria	entire plant	decoction	
	Macaranga sp	sap leaves	Expression	
	Hymenocardia acida	barks and roots	maceration	
	Jatropha curcas	latex of stem or leaves	dilution	spoonful
	Uapaca densifolia	barks	decoction	drink
Fabaceae	*Dalbergia melanoxylon*	leaves	decoction	drink
	Pterocarpus erinaceus	stem barks		
Hypericaceae	*Psorospermum cerasifolium*	green seeds	decoction	drink
Labiaceae	*Ocimum sp*	leaves	tea	drink

Leguminosae	*Cassia hirsuta*	leaves	decoction	drink
Lycopodiaceae	*Lycopodiella cernua*	sporange	decoction	drink
Malvaceae	*Sidaacuta*	fresh leaves	decoction or maceration	drink
	Urena lobata	barks or stems orroots	decoction	drink
Mimosaceae	*Acacia dudgeoni*	barks or stems	decoction	drink
	Parkia biglobosa	barks or stems	decoction	bath
Moraceae	*Ficus sycomorus*	barks or stems	decoction	bath
Myrtaceae	*Psidium guajava*	leaves	decoction or maceration	drink
Sterculiaceae	*Dombeya pentandra*	barks	decoction	drink
	Waltheria indica	stems and leaves	decoction	drink
Vernaceae	*Stachytarpheta indica*	leaves	maceration	drink

Table 2: Extraction yield (%) of essential oils and plant extracts

	Extraction yields (%)			
Plants	EO	AE	EE	HEE
A. conyzoides	0.21±0.02	1.3	11.20	6.64
L. camara	0.08±0.03	5.94	10.33	2.15
P. guajava	0.16±0.03	5.99	9.74	5.07
S. acuta	0.00±0.00	4.5	1.48	3.73

EO = essential oils; AE = Aqueous extract; EE = Ethanolic extract; HEE = Hydro ethanolic extract

Table 3: Quantitative and qualitative chemical composition of the essential oils of *P. guajava*, *L. camara* and *A. conyzoïdes* leaves

Compounds names	RI (HP5-apolar)	RI (Adams)	RI (Carbowax-polar)	Percentage (%)		
				P. guajava	*L. camara*	*A. conyzïdes*
Aliphatic				**8.9**	-	**2.1**
(3Z)-hexenol	595	859	-	0.5	-	-
(n)-hexanol	629	870	1349	<0.1	-	-
benzaldehyde	**966**	**960**	**1530**	**7.8**	-	-
6-methyl-Hept-5-en-2-one	984	985	1456	0.6	-	-
Methyl acetate	1287	1267	-	-	-	2.1
Monoterpenes				**6.2**	**23.9**	**5.8**
α-thujene	801	930	-	-	<0.1	-
α-pinene	938	939	1033	-	1.7	0.7
Camphene	952	954	1073	-	<0.1	1.8
Sabinene	**978**	**975**	**1134**	-	**8.4**	**-**
β-pinene	983	979	1117	-	1.3	-
Myrcene	991	990	1169	-	0.8	-
α-phellandrene	1007	1002	-	-	<0.1	0.7
δ-3-carene	1014	1011	1156	-	1.2	-
α-terpinene	1019	1017	1183	-	<0.1	0.9
*para*cymene	1026	1024	1273	-	<0.1	1.2
Myrcene	990	990	929	<0.1	-	-
Limonene	1031	1029	1202	<0.1	0.7	0.5

1,8-cineole	**1035**	**1031**	**1215**	**5.7**	**6.9**	**-**
(Z)-β-ocimene	1040	1037	1035	-	<0.1	-
(E)-β-ocimene	1050	1050	1046	<0.1	<0.1	-
γ-terpinene	1060	1059	1251	<0.1	<0.1	-
Linalool	1099	1096	1548	<0.1	-	-
cis-hydrate de sabinene	1068	1065	-	-	<0.1	-
terpinolene	1090	1088	1284	-	<0.1	-
Linalool	1101	1096	1562	-	<0.1	-
trans-hydrate de sabinene	-	1098	1556	-	<0.1	-
Camphre	1146	1146	1519	-	0.9	-
Borneol	1166	1169	1684	-	<0.1	-
terpinen-4-ol	1175	1177	-	<0.1	2	-
α-terpineol	1189	1188	1683	0.5	<0.1	-
Sesquiterpenes				**83.9**	**73.8**	**80.8**
δ-elemene	1334	1338	1474	-	<0.1	-
α-copaene	1372	1376	1494	<0.1	<0.1	-
α-2-*epi*-funebrene	1392	1382	-	<0.1	-	-
β-elemene	1387	1390	1609	-	1.2	-
7-*epi*-sesquithujene	1389	1391	1716	<0.1	-	-
italicene	1394	1405	1541	<0.1	-	-
sesquithujene	1404	1405	-	<0.1	<0.1	-
α-cedrene	1413	1411	1572	0.8	<0.1	-
α-*cis*-Bergamotene	1415	1412	1568	<0.1	-	-
***(E)-β*-caryophyllene**	**1422**	**1419**	**1614**	**26.5**	**12,7**	**24.6**
β-Ionone	**1468**	**1430**				**4.7**
β-copaene	1430	1432	1542	<0.1	<0.1	-
sesquisabinene	1443	1459	1720	-	<0.1	-
α-*trans*-bergamotene	1446	1434	1599	0.6	-	-
β-*epi*-santalene	1452	1447	1643	<0.1	-	-
***α*-humulene**	**1454**	**1454**	**1674**	**3.9**	**4.6**	**-**
(E)-β-farnesene	1456	1456	1676	<0.1	<0.1	0.4
9-*epi*-*(E)*-caryophyllene	1458	1466	-	-	<0.1	-
α-acoradiene	1461	1466	1672	<0.1	-	-
β-acoradiene	1466	1470	1666	1.7	<0.1	-
β-10-*epi*-acoradiene	1465	1475	-	<0.1	-	-
γ-muurolene	1471	1479	1691	<0.1	<0.1	-
***γ*-curcumene**	**1473**	**1482**	**1682**	2	**3.8 ***	**-**
germacrene D	1473	1485	1696	-	0.5 *	**41.6**
ar-curcumene	1479	1480	1776	1.5	1.9	-
β-selinene	1480	1490	1708	<0.1	-	-
α-selinene	1486	1498	1713	<0.1	-	-
***α*-zingiberene**	**1488**	**1493**	**1728**	-	**5 ***	**-**
isodaucene	**1488**	**1500**	**-**	**3.7**	-	-
bicyclogermacrene	1488	1500	1734	-	2.3 *	-

α-muurolene	1493	1500	-	<0.1	-	-
(Z)-α-bisabolene	1496	1507	-	3.4	-	-
β-curcumene	1496	1515	1696	-	3.4	-
β-bisabolene	1506	1505	1724	2.9	-	2.9
Cubebol + γ-cadinene	1520	1515	-	-	<0.1	-
δ-cadinene	1502	1523	1755	-	2.2	1.3
γ-cadinene	1509	1513	1682	<0.1	-	-
β-curcumene	1509	1515	1731	1.1	-	-
(Z)-γ-bisabolene	1513	1515	-	0.7	-	1.4
β-sesquiphellandrene	**1521**	**1522**	**1756**	**4.3**	-	-
(E)- γ-bisabolene	1529	1531	1740	3.3	-	-
α-copaene-11-ol	1535	1541	2065	-	<0.1	-
italiceneether	1544	1537	1853	<0.1	-	-
(E)-α-bisabolene	1534	1540	-	0.8	-	-
germacrene B	1547	1561	1825	-	0.4	-
davanone B	1569	1566	1953	-	0.6	-
(E)-nerolidol	**1559**	**1563**	**2030**	**7.2**	**26.4**	**-**
davanone D (isomère 1)	1562	-	1950	-	0.2	-
caryolan-8-ol	1565	1572	-	<0.1	-	-
spathulenol	1576	1577	2113	-	0.6	-
caryophyllene oxide	1580	1583	1962	2.2	0.7	0.5
davanone D (isomère 2)	1585	1585	2030	-	0.6	-
Gleenol	1589	1587	2038	<0.1	-	-
Zingibérenol	1595	-	-	-	-	**3.4**
epoxyde d'humulene II	1596	1608	2034	<0.1	-	-
not identified 1	1600	-	-	-	0.6	-
2,(7Z)-bisaboladien-4-ol	1604	1619	-	<0.1	-	-
not identified 2	1605	-	-	-	0.7	-
epi-cubénol	1624	1628	2044	<0.1	-	-
eremoligenol	1626	1631	-	<0.1	-	-
α-acorenol	1630	1633	2166	0.7	-	-
not identified 3	1630	-	-	-	2.7	-
naphth-1-ol	1635	1641 **	-	-	1	-
caryophylla-4(12),8(13)-dien-5-α-ol	1637	1640	-	1.4	-	-
α-epi-cadinol	1640	1640	2163	<0.1	-	-
α-muurolol	1642	1646	2176	0.5	-	-
not identified 4	1647	-	2419	-	0.9	-
neo-intermedeol	1655	1660	2125	3.1	-	-
not identified 5	1663	-	-	-	0.1	-
β-bisabolol	**1665**	**1675**	**2142**	**8.9**	-	-
not identified 6	1667	-	-	-	0.8	-
α-epi-bisabolol	1674	1684	-	<0.1	-	-
α-bisabolol	1676	1685	2187	2.2	-	-

(2Z,6E)-farnesol	1699	1723	2355	0.5	-	-
Diterpenes					**1.6**	**-**
Phytol	2068	1943	2591	-	1.6	-
Total identified				**99**	**99.4**	**88.7**

**=relatives proportions obtained on polar column; **=retention indices obtained from FFNSC2.L*

Table 4: Phytochemical analysis of plant extracts using qualitative tests

	A. conyzoïdes			*L. camara*			*P. guajava*			*S. acuta*		
Phytochemical compounds	AE	EE	HEE	AE	EE	HEE	AE	EE	HEE	AE	EE	HEE
Alcaloïds	+	+	+	+	-	+	+	-	-	+	+	+
Athocyanins	+	+	+	+	-	+	+	+	+	+	+	+
Flavonoïds	+	+	+	+	+	+	+	+	+	+	+	+
Phenols	-	+	-	+	+	+	-	+	-	-	+	-
Polyphenols	+	-	+	-	-	-	+	-	+	+	-	+
Saponins	-	+	+	-	+	+	+	+	+	-	+	+
Stéroids	+	+	-	-	+	-	-	+	-	+	+	-
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Triterpenes	-	-	+	+	-	+	-	-	+	-	-	+

+ = present; - = absent ; AE = Aqueous extract; EE = Ethanolic extract; HEE = Hydro ethanolic extract

Table 5: Inhibition zone diameters of the essential oils and Gentamicin against the tested bacteria using the disk diffusion assay.

	Bacterial species Inhibition zone diameters (mm)				
Essential oils	*B. cereus*	*E. coli*	*S. enteritidis*	*Shigella spp*	*S. aureus*
A. conyzoides	7.0 ± 0	8.5 ± 0.5	7.3 ± 0.6	7.6 ± 0.5	9.7 ± 0.6
L. camara	-	9.0 ± 1.0	13.0 ± 2.0	11.0 ± 0.5	-
P. guajava	10.6 ± 0.5	11.6 ± 0.5	13.6 ± 2.5	10.6 ± 0.5	12.6 ± 0.5
Gentamicin	33.0 ± 1.0	30.6 ± 0.5	31.6 ± 7.0	37.6 ± 2.5	27.6 ± 5.5

Table 6: Inhibition zone diameters of the crude extracts against the tested bacteria using the disk diffusion assay

	Bacterial species Inhibition zone diameters (mm)				
Extract	*B. cereus*	*E. coli*	*S. enteritidis*	*Shigella spp*	*S. aureus*
AE_{Ac}	12.0 ± 1.0	11.0 ± 1.0	11.3 ± 0.6	11.3 ± 0.6	12.7 ± 2,1
EE_{Ac}	-	-	-	-	-
HEE_{Ac}	-	-	9.3 ± 0.6	-	-
AE_{Lc}	-	-	-	-	-
EE_{Lc}	13.0 ± 2.0	14.3 ± 2.1	12.8 ± 1.6	9.7 ± 0.6	-
HEE_{Lc}	11.0 ± 1.0	12.3 ± 0.6	15.0 ± 3.5	12.3 ± 1.5	11.7 ± 1.5
AE_{Pg}	-	-	14.0 ± 5.3	-	-
EE_{Pg}	11.3±1.1	11.7 ± 0.6	11.7 ± 0.6	10.3 ± 0,58	10.3 ± 0.6
HEE_{Pg}	-	12.0 ± 1.0	16.0 ± 3.6	12.0 ± 1.0	11.3 ± 2.3
AE_{Sa}	-	-	-	-	-
EE_{Sa}	-	9.3 ± 1.5	-	-	-
HEE_{Sa}	10.0 ± 1.0	9.3 ± 0.6	13.0 ± 2.0	10.0 ± 0.0	8.7 ± 1.5

AEAc = Aqueous extract from *A. conyzoïdes*; EEAc = Ethanolic extract from *A. conyzoïdes*; HEEAc = Hydro ethanolic extract *from A.conyzoïdes* ; AELc = Aqueous extract *from L. camara*; EELc = Ethanolic extract from *L. camara*; HEELc = Hydro ethanolic extract from *L. camara* ; AEPg = Aqueous extract from *P. guajava;* EEPg = Ethanolic extract from P. *guajava* ; HEEPg = Hydro ethanolic extract from *P. guajava* ; AESa = Aqueous extract from *S. acuta*; EESa = Ethanolic extract from *S. acuta* ; HEESa = Hydro ethanolic extract from *S. acuta*.

Table 7: Growth Inhibition parameters (MIC, MBC and MBC/MIC) of the EO and Gentamicin on the tested bacteria (mg/mL)

	B. cereus			*E. coli*			*S. enteritidis*			*Shigellaspp*			*S. aureus*		
	MIC	MBC	MBC/ MIC	MIC	MBC	MB C/M IC	MIC	MBC	MB C/M IC	MIC	MBC	MB C/M IC	MIC	MBC	MBC /MIC
A. conyzoïdes	25	25	1	50	100	2	100	100	1	50	100	2	100	100	1
L. camara	25	100	4	25	25	1	25	25	1	12.5	25	2	25	25	1
P. guajava	25	25	1	25	25	1	25	25	1	6.25	25	4	12.5	12.5	1
Gentamicin	0.01	0.03	1	0.03	0.03	1	0.15	0.39	1	0.07	0.31	1	0.15	0.39	4

MIC =Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration

Table 8: Growth Inhibition parameters (MIC, MBC and MBC/MIC) of the non volatile extracts on the tested bacteria (mg/mL).

		B. cereus			*E. coli*			*S. enteritidis*			*Shigella spp.*			*S. aureus*		
Plants	Extracts	MIC	MBC	MBC/ MIC	MIC	MBC	MBC/ MIC	MIC	MBC	MBC /MIC	MIC	MBC	MBC/ MIC	MIC	MBC	MBC/ MIC
A. conizoïdes	AE	100	100	1	50	100	2	25	25	1	50	100	2	25	25	1
	EE	6.25	6.25	1	12.5	12.5	1	12.5	12.5	1	12.5	25	2	25	25	1
	HEE	6.25	6.25	1	6.25	6.25	1	6.25	6.25	1	12.5	25	2	6.25	6.25	1
L. camara	AE	25	100	4	6.25	12.5	2	6.25	6.25	1	6.25	6.25	1	1.56	3.12	2
	EE	3.12	3.12	1	3.12	3.12	1	1.56	1.56	1	1.56	3.125	2	1.56	1.56	1
	HEE	0.78	0.78	1	0.78	0.78	1	0.78	1.56	2	0.78	3.125	4	0.39	1.56	4
P. guajava	AE	0.19	3.12	16	3.12	6.25	2	6.25	6.25	1	1.56	1.5625	1	0.78	3.12	4
	EE	0.78	12.5	16	3.12	6.25	2	6.25	6.25	1	3.12	12.5	4	0.78	12.5	16
	HEE	3.12	12.5	4	3.12	6.25	2	6.25	6.25	1	3.12	3.125	1	3.12	12.5	4
S. acuta	AE	6.25	12.5	2	50	50	1	12.5	12.5	1	6.25	6.25	1	12.5	100	8
	EE	0.78	1.56	2	3.12	3.12	1	3.12	6.25	2	1.56	6.25	4	1.56	6.25	4
	HEE	1.56	1.56	1	3.12	6.25	2	0.78	3.125	4	3.12	3.12	1	3.12	3.12	1

AE = Aqueous extract; EE= Ethanolic extract; HEE= Hydro ethanolic extract;

MIC =Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration

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Manuscript Style Instruction (Optional)

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swis721 Lt BT.
- Page size: 8.27" x 11'", left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.

Format Structure

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

Title

The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Criterion for Grading a Research Paper (Compilation) by Global Journals

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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