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**RESEARCH ARTICLE**

# IN VITRO ANTIOXIDANT ABILITIES AND INHIBITION OF RABBIT MUSCLE LACTATE DEHYDROGENASE BY AQUEOUS EXTRACT OF HANNOA KLAINEANA STEM BARK.

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## *Abstract*

This study was aimed at investigating antioxidant properties and inhibitory effect on Lactate dehydrogenase by aqueous extract of *Hannoa klaineana* stem bark. The bark of the stem was assessed for its antioxidant activity by determining the ability of the extract to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH.) radical, superoxide anion ($O_2^{\cdot-}$) and nitric oxide radicals. The stem bark showed a significant ($p<0.05$) maximal inhibition of $DPPH^{\cdot}$ Radical value of 55.23 ± 1.34% at 10,000 µg/ml of extract compared to ascorbic acid which inhibited 72.30 ± 3.12% at the concentration of 10,000 µg/ml. Maximal superoxide anion ($O_2^{\cdot-}$) inhibition was 103.42 µg/ml at the 1000 µg/ml of extract. The $IC_{50}$ for the stem bark extract inhibition of DPPH and $O_2^{\cdot-}$ anion radical were found to be 103.42 µg/ml and 94.21 µg/ml respectively compared to ascorbic acid and quercetin (8.35µg/ml and 10.87µg/ml). The extract was also found to rapidly scavenge nitric oxide at different time intervals. Also, the $K_m$ and $V_{max}$ of LDH were 27.8 µg/ml and 11.1 µmol/min.mg$^{-1}$ protein respectively. The double reciprocal plot for the aqueous extract revealed a non competitive type of inhibition based on their doses. The inhibition Constant (Ki) was 14.5 µg/ml which indicates high inhibitory capability of the extract on lactate dehydrogenase in dose-dependent manner. Hence, this plant extract could be use as sources of natural antioxidant, enzyme inhibitory agent and as a promising opportunity in designing a novel drug with high efficacy to slow down the rate at which malaria parasite and tumour cells proliferate.



## Introduction:-

The broad use of plants in the treatment of diseases has been a general practice in most developed countries throughout the world however; few of these plants have received adequate scrutiny. Therefore, the increasing desire in therapeutic plants for the management of disease requires information on the antioxidant and inhibitory abilities of the various plants used. Also, *Allium sativum* and *Gossypium arboretum* have been used for the treatment of pathogenesis of situations such as malaria and cancer. Researchers have been showing greater interest in Lactate dehydrogenase because it is an essential therapeutic tool for malaria and cancer. (Rabiu *et al.*, 2013). In addition, scientific investigations have shown that, inhibiting LDH from converting pyruvate to lactate in cancer cells may possibly stimulate mitochondrial function and might also stimulate tumour cells and malaria parasite proliferation (Rabiu *et al.*, 2013).

The selected medicinal plant known as *Hannoa klaineana* belongs to the family Simaroubaceae and the genus is *Hannoa.* It can be used traditionally in Central African countries for the treatment of fever and malaria (François *et al.*, 1998). Also, it can be used locally in South East of Nigeria to treat malaria and cancer.

## Materials and method:-

**Plant Identification and Authentication:-**

The *Hannoa klaineana* plant was critically identified and authenticated in International Centre for Ethnomedicine and Drug Development (InterCEDD) in Nsukka, Enugu State, Nigeria. The specimen was washed to discard impurities and was placed thinly on the flat clean tray (to prevent spoilage by moisture condensation) and permitted to dry at room temperature for seven days (Sofowora 1982). The stem of the plant was pulverized into the form of a powder by the means of an electric mill and stored until the need arises.

**Source of Lactate dehydrogenase:-** Purified L2500-25KU (942 units/mg protein) Lactate dehydrogenase from rabbit muscle was purchased from Sigma Aldrich.

**Methodology:-**

**a) Preparation of extract:-**

The pulverised sample was prepared by cold maceration technique (O'Neill, et al., 1985). The plant material was extracted by refluxing 45g of the specimen in 2.5L of distilled water for three uninterrupted days at room temperature. The sample extracted was filtered by the use of a cotton and whatman #1 filter paper to get the filtrate. Rotary evaporator was used to concentrate aqueous extract. The extract was stored at $4^oC$ until it was used.

**b) *In vitro* Antioxidant assays:-**

- **Qualitative DPPH radical-scavenging using thin-layer chromatography:-**

Qualitative screening for anti-oxidant activity was done using DPPH according to the method of Solerrivasin (2000). Briefly, a thin layer chromatogram of the extract on silica gel plates were developed using aqueous-ethyl acetate (50:50v/v) as mobile phase. DPPH radical test was performed directly on thin layer chromatography (TLC) plates by spraying with DPPH (0.2% w/v) in aqueous to reveal the antioxidant activity of the extracts.

- **Quantitative DPPH radical-scavenging assay:-**

The method of Gyamfi *et al*. (1999) with slight modification by Awah *et al*., (2012) was used to investigate the scavenging potential of DPPH free radicals by the aqueous extract. It involves the use of the extract solution of 2.0 ml at various concentrations dissolve in two-folds was mixed with DPPH (1.0ml). . The positive control used was L-ascorbic acid. The absorbance of the assay was determined at 518nm; The formula used for DPPH radical scavenging activity investigation can be expressed as: $\% \text{ Inhibition} = 100\% \text{ x } \left(\frac{A_o - As}{A_o}\right)$

Where $A_o$ represents Control absorbance, and As represents tested sample absorbance.

- **Superoxide radical ($O_2^{\cdot-}$)-scavenging assay:-**

The determination of superoxide anion radical was done according to the method of Beauchamp and Fridovich, (1971), Martinez *et al*., (2001) and (Awah *et al* 2012). This method involves the use of reaction mixture and incubation of plant extract in fluorescent light. The absorbance of the assay was determined at 560nm. The formula used for superoxide anion radical scavenging activity investigation can be expressed as:

$$\% \text{ Inhibition} = 100\% \text{ x } \left(\frac{A_o - As}{A_o}\right)$$

Where $A_0$ represents absorbance of control while $A_S$ was tested sample absorbance respectively.

- **Nitric oxide radical ($NO^{\cdot}$) Scavenging assay:-**

The methods of Marcocci *et al*. (1994) and (Awah *et al*., 2012) were used to determine Nitric oxide ($NO^{\cdot}$) radical scavenging assay. It involves incubating the reaction mixture and reacted the nitric oxide radical released with oxygen. The absorbance was determined at 546nm.

**c) Enzyme Analysis:-**

- **Lactate dehydrogenase Assay:-**

The method of Bartholmes *et al.*, (1973) was used for Lactate dehydrogenase determination. It involves incubating the reaction mixture to establish a blank rate and determine the absorbance from the spectrophotometer $\Delta A_{340}$/min at $25^oC$. The initial linear portion and protein concentration was determined using this formula:

$$\text{Units/mg} = \frac{\Delta A340/\text{min}}{6.22 \text{ x mg enzyme / ml reaction mixture}}$$

- **Effect of Substrate Concentration:-**

To determine substrate concentration, Sodium Pyruvate was prepared at different concentrations and reacted with 0.2M Tris-HCl, pH 7.3 and NADH. Record at $\Delta A_{340}$/min at 25$^{o}$C for 30secs, 60secs, 90secs, 120secs and 150secs. Determine Vmax and Km.

- **Effect of aqueous extract of *Hannoa klaineana* on LDH activity:-**

The aqueous extract of *Hannoa klaineana* was prepared in 5, 10 and 15µg/ml concentration respectively according to the method of Stephens *et al.*, 1986. One millilitre (1ml) of the extract was taken from each stock and added to the reaction mixture for 5 minutes. Initial velocity studies were determined based on the LDH activity determination method.

## Result and discussion:-

**Dot Blot for DPPH Radical Scavenging Capability of extracts:-**

As shown on the thin layer chromatographic plate in Fig. 3.1 below, the aqueous plant extract significantly scavenged DPPH radical by altering the extract spots from the colour purple to yellow in a dose manner. The more rapidly the intensity of the colour of the spot changes to yellow the higher the anti-oxidant activity. The scavenging capability of DPPH radical increased as follows:

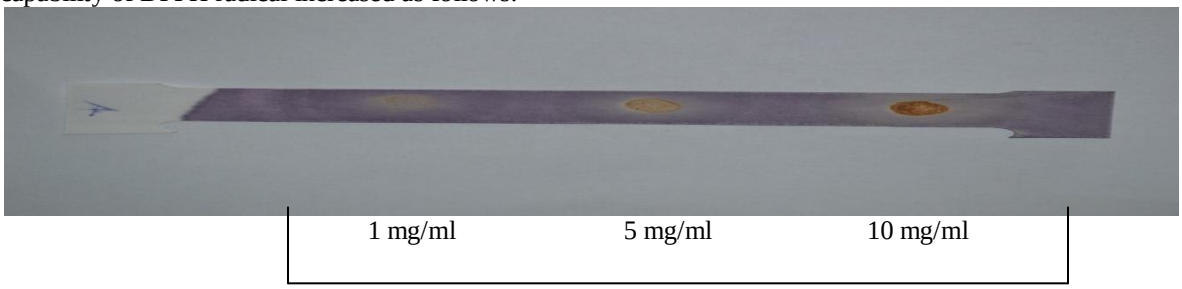


**Plate 1: Dot blot of radical scavenging capacity of *Hannoa klaineana* aqueous extract.**

The presence of secondary metabolites like flavonoid, alkaloid could suggest the ability of the extract to scavenge DPPH radical.

**DPPH radical scavenging activity (%) of aqueous extract of *Hannoa klaineana***

The aqueous extract of *Hannoa klaineana* stem bark showed a significant dose-dependent DPPH radical scavenging capacity (Table 1), inhibiting 55.23 ± 1.34% of DPPH at a concentration of 10,000µg/ml compared to ascorbic acid which inhibited 72.30 ± 3.12 % at a concentration of 10,000µg/ml.

**Table 1: DPPH radical scavenging activity of extracts**

| Extract Concentration (µg/ml) | Inhibition (%) | Ascorbate Concentration (µg/ml) | Inhibition (%) |
|---|---|---|---|
| 10000 | 55.23 ±1.34 | 10000 | 72.30 ± 3.12 |
| 5000 | 44.93 ±4.21 | 5000 | 65.34 ± 2.25 |
| 2500 | 39.34 ±2.56 | 2500 | 63.12 ± 8.34 |
| 1250 | 34.54 ±1.87 | 1250 | 54.78 ± 3.67 |
| 625 | 27.59 ±0.99 | 625 | 48.16± 9.45 |
| 312.5 | 20.72 ±2.36 | 312.5 | 39.23 ± 9.03 |
| 156.25 | 15.54 ±0.65 | 156.25 | 28.14± 8.76 |
| 78.13 | 10.35 ±2.13 | 78.13 | 25.47 ± 3.29 |
| 39.06 | 8.34 ±1.46 | 39.06 | 17.23 ± 1.34 |
| 19.53 | 3.39 ±2.13 | 19.53 | 12.23 ± 2.65 |
| **$IC_{50}$** | 103.42 | | 8.35 |

**Data represent as Mean ± SEM (n=3).**

**Superoxide anion ($O_2^{\cdot-}$) radical-scavenging activity (%) of an aqueous extract of *Hannoa klaineana:-***

The stem bark extract inhibited the formation of reduced NBT in a dose-related manner. As shown below, the maximal superoxide anion ($O_2^{\cdot-}$) inhibition was 57.56 ± 4.23% at the 10,000 µg/ml of extract, compared to quercetin that inhibited 70.13 ± 7.01 %, at 10,000 µg/ml. The $O_2^{\cdot-}$ scavenging effect of the extracts could culminate

in the prevention of $^{\cdot}OH$ radical formation since $O_2^{\cdot-}$ and $H_2O_2$ are required for $^{\cdot}OH$ radical generation. This result supports the report of Robak and Gryglewski (1988), that some phenolic components like flavonoid and catechin could scavenge superoxide anion radical successfully.

**Table 2: Superoxide anion radical scavenging activity of *Hannoa klaineana***

| Extract Concentration (µg/ml) | % Inhibition | Quercetin Concentration (µg/ml) | % Inhibition |
|---|---|---|---|
| 10000 | 57.56 ± 4.23 | 10000 | 70.13 ± 7.01 |
| 5000 | 50.48 ± 0.65 | 5000 | 63.25 ± 3.25 |
| 2500 | 46.79 ± 3.12 | 2500 | 62.48 ± 2.01 |
| 1250 | 43.48 ± 2.14 | 1250 | 58.31 ± 3.21 |
| 625 | 38.55 ± 0.54 | 625 | 53.11 ± 0.34 |
| 312.5 | 36.87 ± 2.16 | 312.5 | 48.27 ± 6.28 |
| 156.25 | 35.23 ± 5.20 | 156.25 | 42.14 ± 1.34 |
| 78.13 | 30.385 ± 3.23 | 78.13 | 39.12 ± 0.28 |
| 39.06 | 22.80 ± 1.40 | 39.06 | 34.45 ± 3.12 |
| **IC50** | 94.21 | | 10.87 |

**Data represent as Mean ± SEM (n=3).**

**Scavenging effect of aqueous extract of *Hannoa klaineana* on Nitric oxide ($NO^{\cdot}$) production:-**

The aqueous extract of *Hannoa klaineana* exhibited good $NO^{\cdot}$ scavenging activity leading to the reduction of the nitrite concentration in the assay medium. The $NO^{\cdot}$ scavenging capacity was concentration dependent with 1000µg/ml scavenging most efficiently. The extract in SNP solution significantly inhibited ($p<0.05$) the accumulation of nitrite, a stable oxidation product of $NO^{\cdot}$ liberated from SNP in the reaction medium with time.

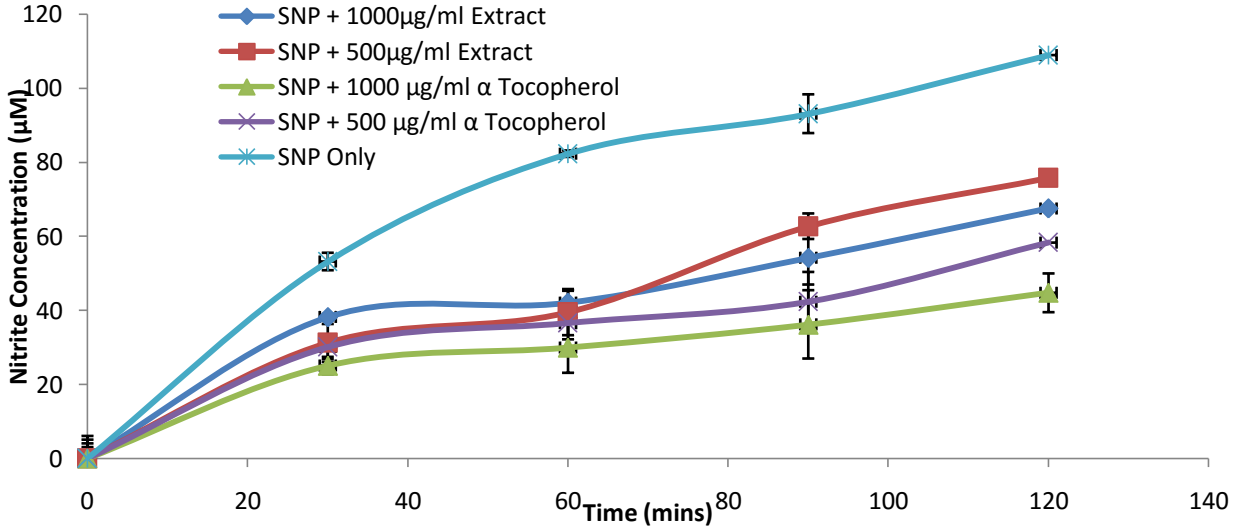


**Figure 1: Effect of *H. klaineana* aqueous extract on the generation of nitrite upon disintegration of sodium nitroprusside (SNP; 5mM) at 25°C.** Each plot represents mean ± SEM (n = 3).

Nitric oxide released from SNP has a strong $NO^{+}$ property which can alter the structure and function of many cellular components (Awah *et al.*, 2012) The aqueous extract of *H. klaineana* in SNP solution decreased levels of nitrite, a stable oxidation product of $NO^{-}$ liberated from SNP. The essential role of nitric oxide in oxidative damage is obviously tangible regardless of its helpful outcome (Beckman and Koppenol, 1996). This could be as a result of the reaction of superoxide and nitric oxide to produce peroxynitrite anion that is capable of breaking down to release $NO_2$ and $^{-}OH$ as a result of its essential characteristics as a capable powerful oxidant (Pacher *et al.*, 2007). The capability of $NO^{-}$ to scavenge free radicals depends on the extract concentration. The extract concentration of 1000µg/ml was most effective compared to other concentrations.

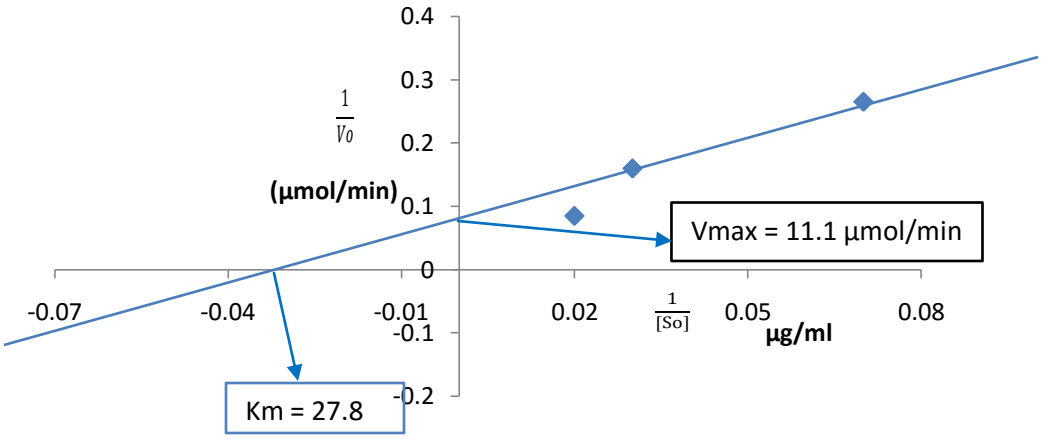


**Figure 2: Lineweaver-Burk's plot of LDH activity against Substrate Concentrations.**
**The result revealed a Km value of 27.8 µg/ml and Vmax value of 11.1 µmol/min. The inhibitor could not alter the Km but there was a variable change in Vmax. The total enzyme concentration was strongly decreased which could be due to the fact that their individual binding effect was not affected by either the inhibitor (extract) or the substrate.**

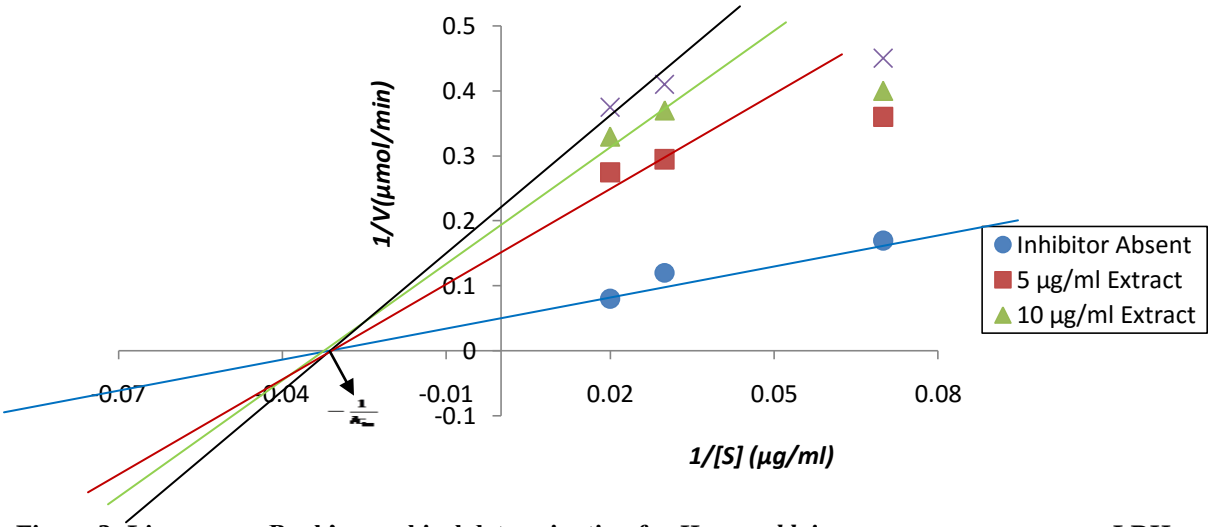


**Figure 3: Lineweaver-Burk's graphical determination for *Hannoa klaineana* aqueous extract on LDH Activity indicating non-competitive inhibition.**

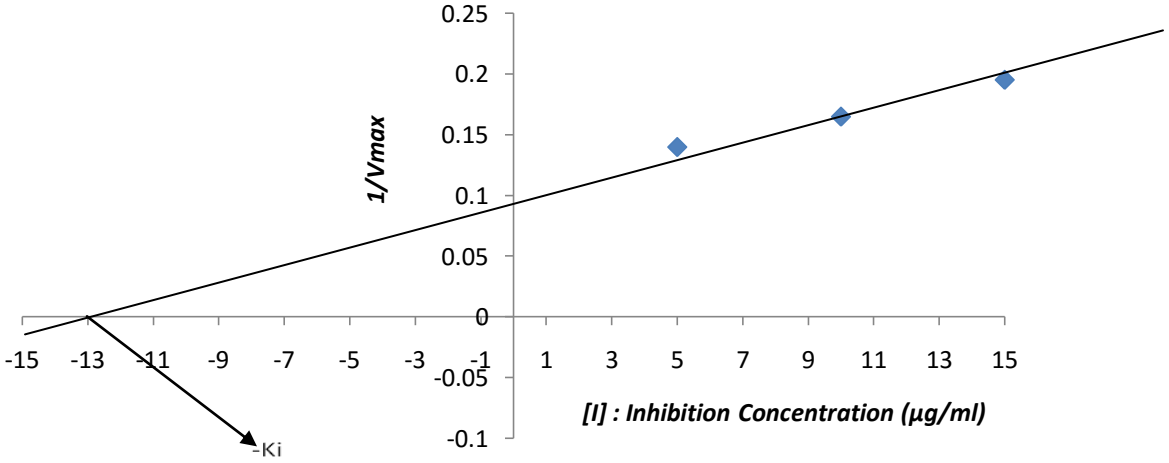

**Figure 4: Graphical determination of *Hannoa klaineana* aqueous extract Inhibition Constant (Ki) indicating 14.5 µg/ml.**

The LDH activity result from aqueous extract of *Hannoa kleineana* suggests a non-competitive inhibition (Figure 3). The inhibitor binds on the enzyme at a site different from that of the substrate, which could be as a result of the extract destroying the enzyme catalytic activity thereby changing the conformational state of the catalytic site and allowing the binding of the inhibitor at different site other than the substrate. The low Ki value of 14.5 µg/ml (Figure 4) obtained from the plant extract expressed on the LDH enzyme demonstrates the high enzyme affinity for the extracts. Since researchers have developed that low Ki value is an indication of high affinity which the enzyme has for the extract.

Hence, the inhibition of this essential enzyme in anaerobic glycolysis (reduction of pyruvate to lactate) may demonstrate a rational and therapeutic approach for the treatment of malaria infection and cancer. From this research, *Hannoa kleineana* aqueous extract displayed a strong inhibition of this experimented enzyme in a dose-dependent manner. Furthermore, this inhibition may provide novel opportunities for selecting and designing a therapeutic drug that may target the enzyme thereby altering carbohydrate metabolism leading to the obstruction of energy production hence, preventing proliferation of malaria parasite and cancer cells.

## Conclusion:-

This research demonstrates that *Hannoa kleineana* aqueous extract possess good antioxidant and enzyme inhibitory capabilities. Hence, it could serve as a medicinal plant capable of scavenging free radicals and also inhibiting LDH by altering glucose metabolism and slowing down the rate of energy production thereby impeding plasmodium infection and tumour cell proliferation.

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