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BIOLOGICAL ACTIVITIES (ANTI-INFLAMMATORY AND ANTI-OXIDANT) OF FRACTIONS AND METHANOLIC EXTRACT OF *PHILONOTIS HASTATA* (DUBY WIJK & MARGADANT).

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Abstract

Background: Bryophytes like other lower plants (non-vascular plants) are not traditionally employed for therapeutic purposes. Hence this study evaluated the *in vitro* anti-oxidant potentials and anti-inflammatory activities of the fractions and methanolic extract of Moss (*Philonotis hastata*, Duby) with a view to studying its biological activities.
Methods: Plant materials were collected, identified, dried, pulverized and extracted with 70% (v/v) methanol to afford methanolic extract (ME). The ME was fractionated using solvent of increasing polarity and followed by phytochemical screening of the extract and fractions. The anti-oxidant potentials (total phenolics and flavonoids), anti-oxidant activities (DPPH-radical scavenging, reductive power, hydroxyl radical scavenging and $ABTS^+$ radical scavenging) and anti-inflammatory activity (red blood stabilizing) of the extract and fractions were investigated.
Results: Phytochemicals detected included alkaloids, steroids and cardiac glycosides. The fractions and methanolic extract exhibited moderate anti-oxidant potentials with various models. The flavonoid contents of the methanol extract and fractions ranged between 1.70 ± 0.05 and 129.70 ± 1.00 mg/g extract RE (Rutin Equivalent) and total phenol content ranged between 1.84 ± 0.62 and 136.25 ± 0.18 mg/g extract TAE (Tannic Acid Equivalent). The reductive power activity ranged between 5.71 ± 0.02 and 40.40 ± 0.30 mg/g Vitamin C equivalent, the hydroxyl radical scavenging (15.54 ± 0.02 to 30.07± 0.29 %), and the $ABTS^+$- radical scavenging activity (11.59 ± 2.85 to 40.50 ± 1.35%). The membrane stabilizing potentials of the fractions and extract (methanol) ranged between 19.19 ± 2.66% and 78.26 ± 2.60%. The activities were comparable to standard anti-inflammatory drugs and chemicals.
Conclusion: It could be concluded that the fractions and methanol extract of *P. hastata* exhibit moderate, potent and appreciable anti-oxidant and anti-inflammatory activities with aqueous and ethyl acetate fractions elicited highest activities. As such, bryophytes possess therapeutic potentials and could be employed in the treatment and management of anti-oxidant and anti-inflammatory disorders.

Key words: *Philonotis hastata*, bryophytes, membrane - stabilizing, flavonoids, anti-oxidant, anti-inflammatory.

Introduction

Reactive oxygen species (ROS) are generated or produced during normal metabolic activities (Padma et al., 2013) and are involved in the pathogenesis of various diseases such as cancer, ageing, coronary heart diseases, arthritis, atherosclerosis, cataract, inflammation, diabetes and neurodegenerative disorders (Maxwell 1995, Diaz et al., 1997, Aruoma1998). Moreover, ROS have been implicated in the activation of matrix metalloproteinase (collagenase) leading to destruction of tissues (Cotran et al., 1994). However, consumption of plant materials such as leafy vegetables, fruits, and tea extracts have been reported to be associated with reduced incidence of these pathologies (Demiray et al., 2009, Su et al., 2011, Selestin et al., 2013).

Bryophytes are small herbaceous plants such as mosses, liverworts and hornworts that grow closely packed together in mats or cushion on rocks, wall, and soil or as epiphytes on the trunks and leaves of forest trees (Goffinet and Williams 2004). Of the three phyla of bryophytes, greatest species divert is found in mosses with up to 15,000 species. Bryophytes play a vital role in regulating ecosystems because they provide an important buffer system for other plants which live alongside and benefits from the water and nutrients that bryophytes collect. Moreover, liverworts synthesize a vast array of volatile oils which they store in unique organelles called “oil bodies”. These compounds impart an often spicy aroma to the plants which seem to discourage animals from feeding on them. Many of these compounds have been demonstrated to possess and exhibit anti-microbial, anti-cancer, pharmaceutical properties (Goffinet and Williams 2004, Mathews 1994, Pojar and Mac 1994). Mosses, studies have shown to exhibit ability to absorb blood, their free from blood contamination, employed as horticultural media, as sources of peat, and as bio-indicator (Rao 1982, Satake et al., 1990, Mouvet et al., 1993).

Literature survey revealed that there was dearth of scientific information on the medicinal potentials and biological activities of fractions and extracts of bryophytes (*P. hastata*). As such, this paper reported the extraction, fractionation, phyto-constituents, anti-oxidant potentials and anti-inflammatory activities of extract and fractions of *P. hastata*. This was with a view to investigating the possibility of employing the plant for therapeutic purposes.

Materials and Methods

Plant Materials: Collection and Identification of Plant Material.

Fresh *Philonotis hastata* (voucher No.17407) were collected from the walls of Oduduwa Hall and Reforestation Garden, Obafemi Awolowo University, Ile-Ife. The identification and authentication were carried out by Dr. A. M. Makinde (one of the authors), Department of Botany, Obafemi Awolowo University, Ile-Ife. The plants were washed free of dust and sand with running-water, rinsed with distilled water and then oven dried at 60^o C. The dried plant material was then pulverized with hand operated grinding machine to fine powder.

Reagents and Chemicals

All the reagents used in this study were of analytical grade and were procured from various chemical companies. 2, 2, diphenyl-1-picrylhydrazyl (DPPH), 2,2,-azinobis(ethyl) benzothiazoline-6-sulphonic acid ($ABTS^+$), Trolox, Tannin, Quercetin were from Sigma-Aldich, Folin-Ciocalteu's phenol reagent (Fulka Biochemika). Paracetamol (standard anti-inflammatory drug) and Vitamin C (Ascorbic acid) were purchased from Campus Pharmacy at Obafemi Awolowo University, Ile-Ife, Nigeria.

Collection of Fresh Bovine Blood

Fresh bovine blood was collected from the Araromi Abattoir, along Ede Road, Ile-Ife, into an anticoagulant (containing 3.8% trisodium citrate) and was mixed gently to avoid lysing. The blood was transported in ice- bucket to the Laboratory for further processing.

Extraction and Fractionation of Plant Material

Powdered plant material (1.2 kg) was extracted with 8.5L of 70% (v/v) methanol for 72 hr and filtered. The residue was re-extracted 5 more times until the filtrate became colourless. The filtrates were combined and then evaporated to dryness under reduced pressure using Edwards's High vacuum pump at 35^o C to yield greenish syrupy residue termed methanolic extract (PHME). The extract was dissolved in 100 ml of hot distilled water and then partitioned sequentially with n-hexane (50 ml x 5), ethyl acetate (50 ml x 5) and n- butanol (50 ml x 5). The fractions were concentrated to dryness using Edman's vacuum evaporator at 40^oC to yield hexane fraction (HF), ethyl acetate fraction (EAF), n-butanol fraction (BF) and aqueous fraction (AqF). The extract and fractions were phytochemically screened for the presence of alkaloids, saponins, flavonoids, tannins, cardiac glycosides, triterepenoids, carbohydrates, proteins according to earlier procedures (Oyedapo et al., 1999).

Preparation of Standard Drugs

Paracetamol tablets (250 mg) a product of Meyer and Baker, Ikeja, Lagos were powdered in a mortal using pestle. The powdered tablets were weighed and dissolved in 20 ml of distilled water to give a concentration of 0.5mg/ml.

Biochemical Analysis
(a) Estimation of Total Phenol Concentrations.

The estimation of total phenol was carried out using the Folin - Ciocalteu's phenol reagent reaction method as reported by Singleton et al. (1999). The concentrations of phenols were extrapolated from the standard tannic acid calibration curve and expressed as mg/g extract tannic acid equivalent.

(b): **Estimation of Flavonoid Concentration.**

Estimation of flavonoid concentration was carried out using the aluminum chloride ($AlCl_3$) reaction method as reported by Sun et al. (1999). The concentrations of flavonoid in the fractions and extract were obtained by extrapolation from the rutin standard calibration curve and expressed as mg/g extract rutin equivalent.

Biological Assays
(a): Assay of DPPH - Radical Scavenging Activity: The assay of DPPH- radical scavenging activity was carried out according to the reported method of Blois (1985) as modified slightly by Cakir et al. (2003) with Quercetin as standard drug. The percentage DPPH – radical scavenging activity was estimated according to the expression $\{(Abs_{control} - Abs_{test})/Abs_{control}\} \times 100\%$., where Abs 571nm is absorbance at 517 nm.
(b): Reductive Power Assay: The assay was carried out according to a procedure that was based on the earlier methods of Yen and Chen (1995) and Aina and Oyedapo (2013) with slight modifications using ascorbic acid as reference drug. The increase in absorbance of the tested compounds indicated increase reducing power of the compounds.
(c): Assay of inhibition of Hydroxyl Radical Activity: The assay was carried out according to the procedure that was based on those earlier reported procedures (Luo et al., 2010; Aina and Oyedapo 2013). The percentage scavenging activity was estimated as: $\{Abs_{control} - Abs_{test}\}/Abs_{control} \times 100\%$, where $Abs_{control}$, and Abs_{test} are absorbance at 532 nm of the control and test respectively.
(d): Assay of $ABTS^+$-Radical Scavenging Activity: The assay of $ABTS^+$ -radical scavenging activity was carried out according to the method of Re et al. (1999) using Trolox and 7mM $ABTS^+$/7mM Potassium persulphate as reference drug and substrate respectively. The percentage $ABTS^+$ scavenging activity was calculated as: $[Ao - (As - Ab)]/Ao$, where Ao (Abs_{734nm} of $ABTS^+$ without the sample), As (Abs_{734nm} of the ABTS with the sample), and Abs (absorbance of the sample without $ABTS^+$).
(e): Assay of Membrane Stabilizing Activity: The membrane stabilizing activity assay was carried out using 2% (v/v) bovine erythrocyte suspension while acetaminophen served as a standard drug as earlier reported (Oyedapo et al., 2010). The percentage membrane stability was estimated using the expression:

$$\text{Percentage Membrane Stability} = 100 - \frac{\{\text{Abs test drug - Abs drug control}\}}{\{\text{Abs of blood control}\}} \times 100$$

where the blood control represents 100% lysis or zero percent stability.

Statistical Analysis

Each value represented the mean ± SEM of 4 consistent readings. The significance of the differences between controls, tests and fractions were analyzed using Student's t-test and analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be statistically significant.

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Results and Discussion

In continuation of our series of investigations into the biological and therapeutic potentials of extracts and active principles of lower plants (Bode and Oyedapo 2011, Aina and Oyedapo 2013), the current study reported the isolation, phytoconstituents, fractionation and anti-oxidant potentials and anti-inflammatory activities of fractions and extract of *Philonitis hastata.* This was with a view to studying its potential biological activities.

Phytochemical screening of methanolic extract of *P. hastata* (Duby) revealed the presence of alkaloids, cardiac glycosides, steroids, flavonoids and xanthoproteins. Various studies have reported that saponins and flavonoids possess potent, significant and appreciable anti-inflammatory and anti-oxidant activities (Mohammad et al., 2004, Aquila et al., 2009, Oyedapo et al., 1999, 2010, Akinpelu et al., 2012). The roles of medicinal plants in disease prevention or control have been attributed mainly to the anti-oxidant potentials of their constituents usually a wide variety of polyphenolic compounds (Demiray et al., 2009). Polyphenols (flavonoids) are known to possess multifunctional potentials such as the ability to act as reducing agents, anti-cancer, anti-microbial activities oxygen quenchers in addition to their hydrogen donating properties (John and Grohmann 2001, Aberoumand and Deokute 2008, Demiray et al., 2009; Iyawe and Azih 2011; Bode and Oyedapo 2011). The extraction of 1.2 kg of dried *P. hastata* (Duby) yielded 40.025 g of methanolic extract which was 3.34% of starting material, hexane fraction (HF) 2.85 g, ethyl acetate fraction (EAF) 1.22 g, n-butanol fraction (nBF) 14.01g and aqueous fraction (AqF) 9.14 g. Further analysis of the fractions and extracts for flavonoid and phenolic contents is represented in Table 1.0.

The DPPH assay provides information on the ability of the test compounds to donate their hydrogen atoms. It is a stable free radical which accepts an electron or hydrogen radical and is widely employed to investigate radical scavenging activity. Figure 1 is the DPPH- radical scavenging activity profiles of methanolic and fractions of *P. hastata* and Quercetin. It was observed that the extract (methanol) and fractions (hexane, ethyl acetate, n-butanol aqueous) did not exert DPPH- radical scavenging activity. Earlier studies have shown that some compounds did not elicit DPPH-radical scavenging activity but exhibit potent and appreciable $ABTS^{+}$ radical scavenging activity (Wang et al., 1998, Re et al., 1999).

The reducing capacity of a compound serves as indicator of its potential anti-oxidant capacity (Wong et al., 2006). The reducing ability of a compound depends on the presence of reductants that demonstrate anti-oxidative potential by breaking the free radical chain and donating a hydrogen atom (Duh et al., 1999, Deepa et al., 2013). The reductive anti-oxidant potentials of the fractions and methanolic extract of P. hastata were expressed as equivalent of Vitamin C (Table 2). It was observed that methanolic extract exerted highest reductive anti-oxidant potential, followed by AqF and ethyl acetate in that order. The methanol extract and fractions of *P. hastata* elicit potent and appreciable reducing capabilities which have health implications and benefits.

Table 1: Flavonoid and Phenolic Contents of Fractions and Extract of *P. hastata*.

Extract/Fraction	Phenolic	Flavonoid
Methanol	136.25 ± 0.18	129.76 ± 1.00
n-Hexane	1.84 ± 0.62	1.70 ± 0.50
Ethyl acetate	9.04 ± 0.56	8.51 ± 0.52
n- Butanol	28.08 ± 0.36	14.01 ± 1.73
Aqueous	36.10 ±1.46	17.46 ± 2.65

Each value represented the mean ± SEM of n=5 readings. The concentrations of phenol and flavonoid were expressed as mg/g extract tannic acid equivalent (TAE) and mg/g extract rutin equivalent (RE) respectively.

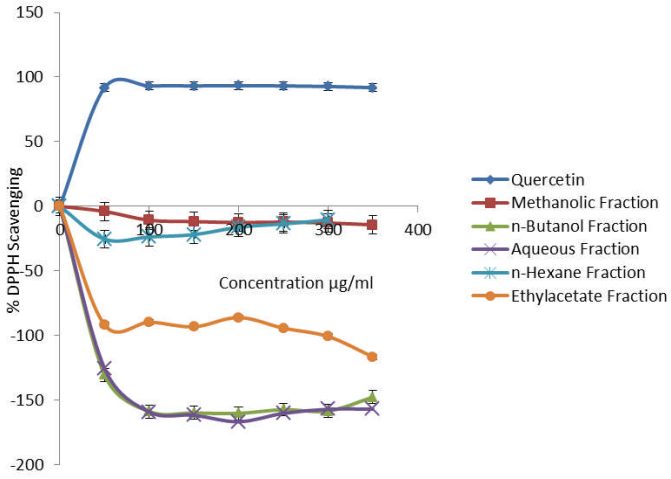

Figure 1: Percentage DPPH- radical scavenging activity of quercetin (standard), extract (methanol), fractions (hexane, ethyl acetate, n-butanol and aqueous) of *P. hastata*.
Each value represented mean ± SEM (n=3).

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Table 2: Reductive Potentials of Fractions and Extract of *P. hastata*.

Extract/Fraction	Reductive Antioxidant Potential
Methanolic	40.40 ± 0.03
n-Butanol	18.93 ± 0.02
Aqueous	26.79 ± 0.02
Ethyl acetate	31.43 ± 0.02
n-hexane	5.71 ± 0.00

Each value represented mean ± SEM (n=3).The reductive power was expressed as vitamin C equivalent (mg Vitamin C/gextract or fraction).

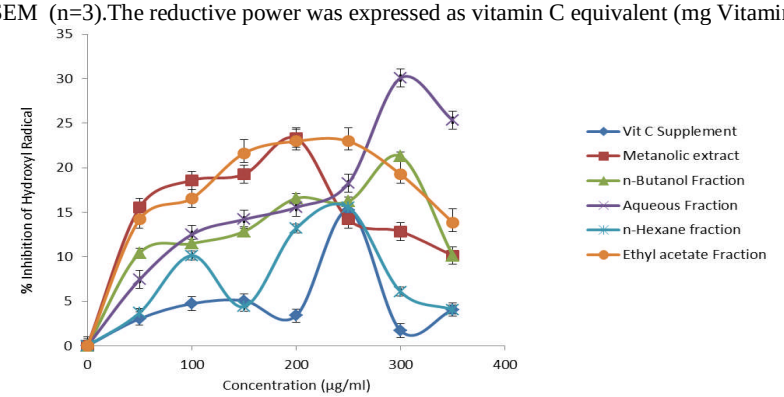

Figure 2: Percentage Inhibition of Hydroxyl Radical Activities of Vitamin C, methanolic extract and its fractions. Each value represented mean ± SEM (n = 3).

Hydroxyl radical scavenging is an important anti-oxidant activity because of very high reactivity of the OH radical which enables it to react with a wide range of molecules finding in living cells such as sugars, amino acids, lipids, nucleic acids and proteins (Stohs and Bagchi 1995; Iyawe and Azih 2011). In Figure 2 is the hydroxyl radical scavenging activity profile of the extract (methanol) and fractions (n-hexane, ethyl acetate, n-butanol and aqueous) with vitamin C as standard. The OH radical scavenging activity of the extract and fractions was concentration dependent. It was observed that the aqueous extract gave the highest activity followed by methanol. The activities of the extract and fractions were comparable to that of Vitamin C and slightly higher at higher concentrations. The maximum percentage Inhibition of hydroxyl radical were methanolic extract 23.31 ± 4.00, hexane 15.54 ± 0.00 and ethylacetate fractions 22.97 ± 8.50. The $ABTS^{+}$ assay is a popular, sensitive and reproducible technique that is employed to evaluate the anti-radical power of extracts through donation of hydrogen atom to form non-radical molecule (Ak and Gulan 2008). The $ABTS^{+}$ radicals are more reactive, its reaction involves an electron transfer process and bleaching of ABTS cation has been employed extensively to evaluate the anti-oxidative capacity (Schlesier et al., 2002). The extract and fractions of *P. hastata* exhibited $ABTS^{+}$-radical scavenging activity with Trolox as standard (Figure 3). The activities of the fractions were higher than those of methanolic with butanol fraction exhibiting the highest activity and comparable to that of standard drug (trolox). The percentage $ABTS^{+}$- radical scavenging activities of the methanolic extract, hexane fraction and ethylacetate fraction have maximum activity of 17.16 ± 0.00, 11.59 ± 2.85 and 25.81 ± 1.03 respectively. The radical scavenging activity was concentration dependent.

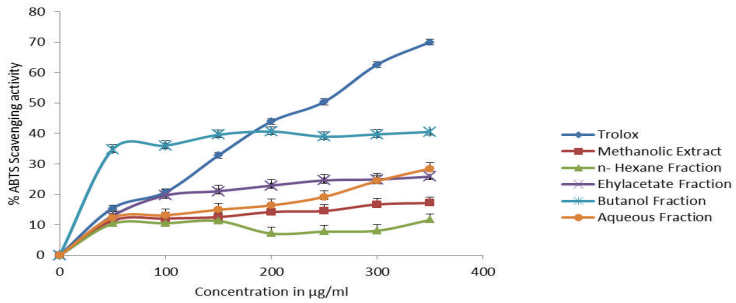

Figure 3: Percentage $ABTS^{+}$ Radical Scavenging Activities of Trolox (standard), methanolic extract and its fractions. Each value represented mean ± SEM (n = 3).

Inflammation is a complex biological phenomenon by which body responses to external stimuli (infections and injuries) (Sadique et al., 1989, Gorzalczany et al., 2011). It is characterized by redness (rubor), heat (calor), swelling (tumor), pain (dolor), dysfunction of the organs involved (lease) and increased blood flow to the affected tissues and organs (Pearson 1972). In many inflammatory disorders, there is excessive activation of phagocytes, production of O^{2-} $\cdot OH$ radicals as well as non-free radical species (H_2O_2) which are capable of damaging tissues either directly or indirectly. The released or generated O^{2-} initiates lipid peroxidation resulting in membrane destruction/damages which then provoke inflammatory

response by the production of mediators and chemostatic factors (Lewis 1989). Various models have been proposed and employed to investigate the efficacy of anti-inflammatory drugs or agents (Oyedapo et al., 1999, Seletin et al., 2013, Kwang-Ho et al., 2008). The biochemical effects exhibited by anti-inflammatory agents or extracts included uncoupling of oxidative phosphorylation, stimulation and or inactivation of phosphatases, red blood membrane stabilization as well as inhibition of denaturation of albumin (Sachin et al., 2010, Sadique et al., 1989, Oyedapo et al., 2010). In this study, red blood membrane stabilization exposed to heat and hypotonic induced lyses technique was employed to investigate the anti-inflammatory activities of extract and fractions of P. hastata. The membrane stabilizing potentials of the extract (methanol) and fractions (n-hexane, ethyl acetate, n-butanol, aqueous) and acetaminophen are shown in Figure 4. It was observed that ethyl acetate fraction exhibited the highest membrane stabilizing activity and methanol the least. The maximum red blood cell membrane stabilizing activities was 19.19 ± 2.66, 59.42 ± 2.73 , 78.26 ± 26 for methanolic extract, hexane and ethylacetate fractions. The percentage red blood cell membrane stability of acetaminophen increases with increase in concentration while those of methanolic extract and its fractions increase at certain concentration and then decreases. The activities were comparable to that of acetaminophen a non-steroidal anti-inflammatory drug. The response of the red blood cells to the extract and fractions was both monophasic and biphasic and concentration dependent. The mode action of the extract and fractions might not be unconnected with binding of the phytochemicals to the erythrocyte membranes couple to the alteration of the cell surface charges. Since red blood cell membrane contains high amount of polyunsaturated fatty acids and vulnerable to oxidative stress. Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids. Lipid peroxidation inactivates cellular components and plays a key role in oxidative stress in biological systems by cell membrane disruption and cell damage (Barrera et al., 2008). Studies have revealed that saponins and flavonoids elicit potent, significant and appreciable membrane stabilizing effect both *in vivo* and *in vitro* (Oyedapo et al., 2010; Akinpelu et al., 2012). Moreover, tannins and saponins bind covalently to cations thereby stabilizing the molecules and biological membranes (El-Shabrany et al., 1997). The abilities of the extract and fractions to scavenge free radicals and bind iron were supported by stabilization of red blood cell membrane exposed to both heat and hypotonic induced lyses.

The results of this study revealed that the bioactive compounds in the extract and fractions of *P. hastata* possess polar characters since they are water soluble. Studies have demonstrated that methanol, ethyl acetate and water soluble extracts exhibit broad spectrum of biological activities. Polyphenols are important class of plants phytochemicals because of their numerous hydroxyl groups that confer free radical scavenging activity (Yang et al., 2009; Bode and Oyedapo 2011). Flavonoids, alkaloids and cardiac glycosides in the extract are indicative of their anti-oxidant, anti-bacterial, analgesic and antinociceptive activities. Evaluation of phenol and flavonoid contents showed that the phenolic fractions represent major constituents of P. hastata.

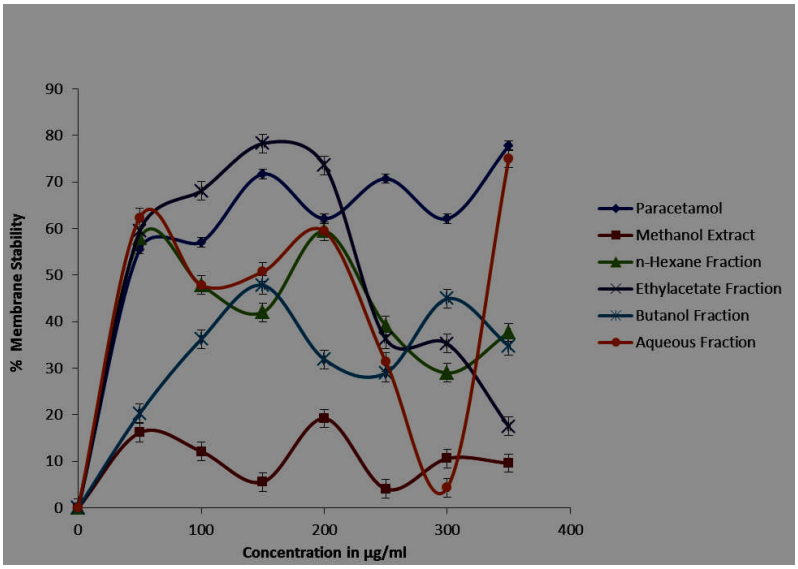

Figure 4: Percentage red blood cell membrane stabilizing activities of Paracetamol (standard), methanolic extract and its fractions. Each value represented mean ± SEM (n = 3).

Doubtlessly, the results of this study could be attributed to the presence of the polyphenolic compounds. The biological activities of these compounds in the treatment and management of oxidant and inflammatory related disorders are not provided by a single group of compounds. Some of these molecules might be acting synergistically to attenuate the bioactivity and efficacy of the bioactive constituents.

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