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Evaluation of phytochemical constituents and *in vivo* anti-inflammatory activity of methanol extract and solvent fractions of *Phyllanthus discoideus* stem bark

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Abstract
Phyllanthus discoideus (Phyllanthaceae) is a tropical tree commonly called Atara in Ebonyi state Nigeria. Its bark is used in indigenous medicine for the treatment of toothache, post-partum pain, leg ulcer, epilepsy and infection. The aim of this study is to evaluate the phytochemical constituents and carry out *in vivo* anti-inflammatory effect of the methanol crude extract and solvent fractions of stem bark of *P. discoideus*. The stem bark of *P. discoides* was collected, identified, prepared and processed into powdered form. Cold maceration extraction method was done using methanol, subsequently, the crude extract was fractionated using solvent-solvent method; n-hexane, ethyl acetate, n-butanol and water were used successively in order of polarity. Phytochemical screening was carried out both on the crude extract and the fractions. Egg albumen and carrageenan methods were used for anti-inflammatory studies using diclofenac as positive control in both methods. From the result, Alkaloid, flavonoids, tannin, saponin, phenolic compound, steroid and carbohydrate were found present. N-butanol has highest anti-inflammatory activity with percentage inhibition of 42.7% and 74.24% at the doses of 300 mg and 600 mg respectively. Other fractions also have relatively good activity compared to the positive control (diclofenac). Therefore, *P. discoideus* stem bark showed a good anti-inflammatory activity which can further be characterized and isolated for management of inflammatory disorders.

Keywords: *Phyllanthus discoideus*, anti-inflammatory, fractions, phytochemical

Introduction
Healing with medicinal plants is as old as mankind itself (K. Lamar. 2014) [23]. The connection between man and his research for drug, in nature dates from the far past, of which there is ample evidence from various sources: written documents, preserved monuments, and even original plant medicines (K. Kaur *et al*. 2014) [24]. Inflammation serves to remove the original source of cell injury, remove necrotic cells and tissues that have been harmed by both the initial insult and the inflammatory process, and start the repair process for injured tissues (Chen *et al*, 2018) [7]. Inflammation could be classified into acute or chronic type with respect to the severity and duration of the inflammation (Zhang *et al.*, 2019; Fritsch and Abreu, 2019) [25, 26]. Acute inflammation is immediate adaptive tissue response to noxious stimuli which can result in chronic inflammation when not controlled (Zhou, Hong & Huang, 2016) [27]. Management of inflammation with the use of traditional medicinal plants has provided an alternative to conventional orthodox therapies for numerous ailments, particularly when suppression of inflammation is expected (Fritsch & Abreu, 2019) [26]. The study of natural molecules from medicinal plants in consort with pharmacological and ethno botanical information have contributed significantly in the improvement of the traditional compounds (Tasneem *et al.*, 2019) [28]. It is generally believed that the active constituents contributing to these protective effects are the phytochemical, anti-inflammatory constituents and minerals (Gill, 2017) [29]. Currently available orthodox anti- inflammatory drugs block both enzyme activities and relief symptoms, but still have serious side effects (Verma, 2016) [30]. It is, therefore, essential to administer anti- inflammatory drugs with lesser side effects. In the study of the activities of phytochemicals in medicinal plants and subsequent identification and isolation of their extracts with proven anti- inflammatory activity, the use of various *in vivo* models and *in vitro* assays of inflammation study have been adopted. *Phyllanthus discoides* is a tree in the family of phyllanthaceae.

This species is distributed in the coastal area of the Eastern Cape, South Africa, tropical Africa, as far as Senegal in West African. The following synonyms; The stem is usually straight with rough, flaking bark which is greyish brown on top and reddish beneath, and the inner part of the fruit is dark metallic blue-green, giving rise to the name bushelled pea cock berry (Osakwe I.I; 2004) [31]

Fig 1: *Phyllanthus discoideus* in Izza Local Government Area of Ebonyi State Nigeria.

2. Material and Methods

2.1 Materials

Methanol, Ethyl acetate, N-butanol, N-hexane, from BDH Chemicals Ltd., Poole England. Distilled water from Pharmaceutical chemistry laboratory, Enugu State University of science and Technology. Ferric chloride, Mayer's reagent, Hager's reagent, 2% hydrochloric acid, chloroform, concentrated H_2SO_4, acetic anhydride, dilute HCL, 5% $FeCl_3$, lead acetate solution, KOH solution in ethanol, 10% NH_3, Chloroform, Sodium hydroxide, EDTA, 2 M $CaCl_2$, pure sample of diclofenac from Sarafica industrial limited Victoria island Lagos. Nigeria. *P. discoides* powdered bark sample, conical flask, Elemeryer flask, Electric weighing balance, Muslim cloth, filter paper, hand gloves, measuring cylinder, macerating bottle, glass funnel, cotton wool, separating funnel, glass stopper, retort stand, Spatular, test tube, syringe, metal animal cage.

2.2 Methods

Collection and identification of *P. discoides*

The stem bark of *P. discoides* was collected at orinte Ndufu Amuzu, Ezza South Local Government Area, Ebonyi state, Nigeria. It was identified and assigned a Voucher Number; INTERCEDD/088 by a taxonomist, Mr. Ozioko.

Preparation of *P. discoides* stem bark

The stem bark of *P. discoides* was cut into small pieces, shade dried under regulated room temperature (25-30 °C) for 28 days. The dried bark was properly selected to avoid contamination and was pulverized into coarse powdered form using a mechanical grinder.

Extraction by cold maceration method

A sample of 500 g of powdered bark of *P. discoides* was weighed into two clean empty, each bottle was properly labeled. Using a calibrated measuring cylinder, 2.51 of methanol solvent was dissolved to each of the bottle and the bottles were stoppered and agitated at 2 hrs intervals for 72 hrs.

Fractionation (Liquid-liquid fractionation)

Fractionation was done in order of increasing polarity (n-hexane, ethyl acetate, and butanol) by mixing equal volume of the fractionation reagents (n-hexane, ethyl acetate, or butanol). The dried methanol fraction, was mixed with 150 ml of distilled water and macerated with n-hexane in parts using 150 ml each, seven times. The n-hexane soluble fraction was filtered and evaporated on water bath at 40 °C to afford n-hexane soluble fraction. The n-hexane insoluble fraction was then macerated with ethyl acetate in parts using 150 ml each, seven times to obtain the ethyl acetate soluble fraction. It was then filtered and evaporated to dryness on water bath at 40 °C. The ethyl acetate insoluble fraction was macerated seven times in parts using 150 ml of butanol each. The butanol soluble fraction was also evaporated to dryness on water bath at 40 °C. The extracts and the fractions were stored in an extract/fraction bottles.

Acute toxicity study (Lorke's method)

Phase 1

Nine animals were grouped into three and properly labelled A1, A2, and A3 and were administered 10 mg/kg, 100 mg/kg, and 1000 mg/kg respectively. The experimental animals were observed for 24 hrs to monitor their behaviours and mortality.

Phase 2

Three animals were used, one animal representing each group labeled B1, B2, and B3. Crude extract was administered at dose of 1600 mg/kg, 2900 mg/kg and 5000 mg/kg respectively. They were observed for 24 hrs for lethal effect.

Anti-inflammatory study

Egg albumin model

Wistar rats (110) weighing between 100-180 g were selected (both sex) and randomly divided into seven different groups which were labelled 1-7 and the first five groups contained 10 rats per groups while the positive and negative control have five rats per group. Each five group (extracts and fractions) were subdivided into two subgroups A and B (1A-1B, 2A-2B, 3A-3B, 4A-4B, and 5A-5B). "A" group were administered 300 mg/kg dose while" B" were administered 600 mg/kg both were used in the two models (Egg albumen and carrageenan induced inflammation) aim at evaluating anti-inflammatory activity of *Phyllanthus discoides* on two inflammatory agent (Egg albumen and carrageenan) induced oedematous paw.

Egg albumen model

Group 1 (A and B)

The total methanol extract doses of all the laboratory rats in group 1 were calculated, they were dissolved at 1:9 ratio in 10 fold dilution using DMSO and water as a vehicle in a clean test tube as a solution. This was shaken before administration of different millilitres of doses to the laboratory rats per oral.

The circumference of various laboratory rats was taken before inducing the inflammation using plethysmometer. The whole rats in both group 1A and 1B were injected with 0.2 ml of egg albumen using 1ml syringe at their plantar region of left hind paw respectively to induce paw oedema.

After 30 minutes of post administration of inflammatory (egg albumen), the circumferences were taken again. Animals in

group 1A were given 300 mg/kg of their respective doses of the methanol crude extract using gavage per oral. Then group 1B were administered 600 mg/kg using gavage per oral. The animals were monitored hourly for four hours. The same procedure was used for group 2 (A and B), 3 (A and B), 4 (A and B) 5 (A and B)

Group 6 (a and b) positive control

The circumference of various laboratory rats was taken with plethysmometer prior to induction of inflammation. The rats in both groups were injected with 0.2 ml of egg albumen using 1ml syringe at their plantar region of left hind paw respectively to induce paw oedema.

After 30 minutes of post administration of inflammatory (egg albumen), their paw circumferences were taken. The animals were orally treated with diclofenac solution at 50 mg/kg using gavage. The whole animals were monitored hourly for 24 hours.

Group 7 (Negative control)

Inflammation was induced using egg albumin and carrageenan respectively and the animals were treated with 10 ml of normal saline.

Carrageenan model

0.1% carrageenan solution was prepared using distilled water.

GROUP 1(A and B)

The animals were administered 0.2 ml of o.1% carrageenan solution using 1ml syringe at the plantar region of the left hind paw of the wistar rats to induce inflammation. After 30 minutes of post administration, group 1A were given 300 mg/kg of their respective doses of the methanol crude extract of using gavage per oral. Group 1B were administered 600 mg/kg doses using gavage per oral. The whole animals were monitored hourly for four hours. The paw size was measured using plethysmometer. The same procedure was applied for group 2 to 5. Thereafter, percentage edema inhibition was gotten using the following formula;

$Pei = (C_0 - C_t)/C_0 \times 100$

Where C_t -is the mean oedema in treated groups while C_0 is the mean oedema in control group

Statistical analysis

The data obtained was analysed and the significance of difference between the control and treated groups determined using one-way analysis of variance (ANOVA) followed by Dunnett's t-test (comparing all the test groups against the positive control). P-values less than 0.05 were considered to be statistically significant.

3. Results

Table 1: Yield of P. discoides extraction and fractionation

Sample	Mass of powdered sample (g)	Mass of crude extract/ fractions (g)	% yield = ma/mb x 100
Methanol crude extract	500	33	6.6
N-hexane fraction	27	2	7.4
Ethyl acetate fraction	27	14	51.6
N-butanol fraction	27	4.5	16.7
Water fraction	27	6.5	24.1

Table 2: Result for phytochemical analysis

Samples	Methanol extract	N-hexane fraction	Ethyl acetate fraction	N-butanol fraction	Water fraction
Tannins	+	-	++	-	-
Alkaloid	+	-	++	-	-
Phenols	+++	-	+	++	+
Steriods	+	+	-	-	-
Saponin	++	-	-	++	-
Carbohydrate	++	-	-	+	+++
Flavonoid	++-	-	+++	+	-

Key:+=present, - =absent

Table 3: Result of acute toxicity study (ld50) of methanol extract of *P. discoides* bark

Groups	Dose (mg/kg)	Mortality rate
Phase 1		
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3
Phase 2		
Group 1	1600	0/3
Group 2	2900	0/3
Group 3	5000	0/3

Table 4: *In vivo* test result of crude extract and fractions of *P. discoides* bark on egg albumen induced paw edema in rat

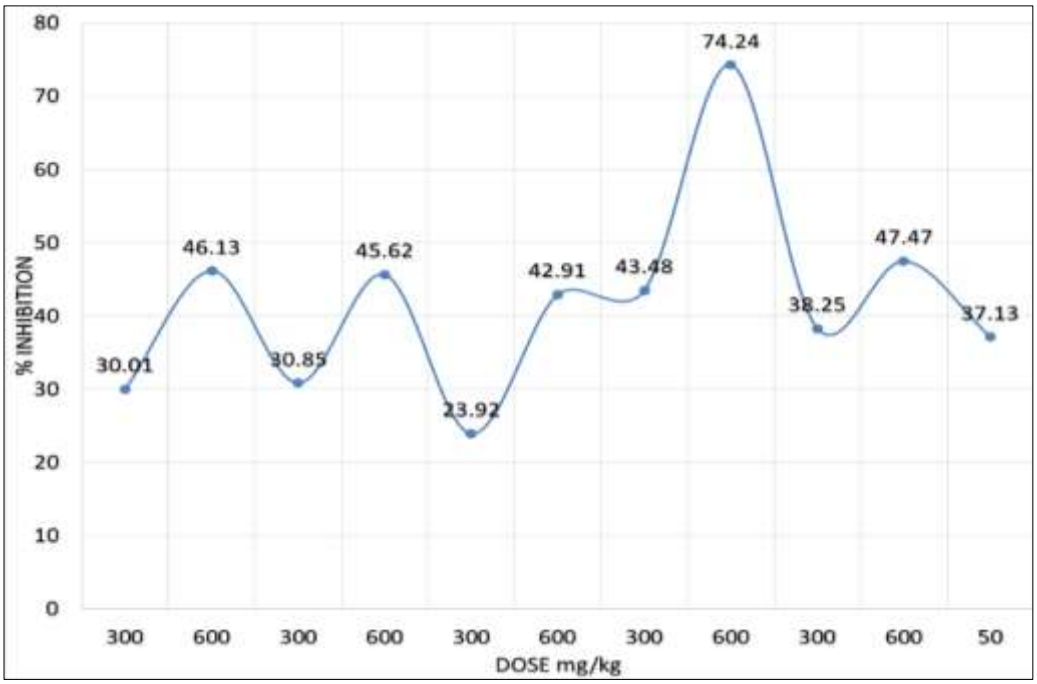
Sample	Dosage mg/kg	0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	24 hrs	% inhibition
Negative control	10 ml NS	10.72±0.60	10.73±0.28	10.65±0.45	11.16±0.45	11.18±0.23	11.73±0.09	11.80±0.04	-
Methanol extract	300a	10.85±0.31	9.19±0.30	7.56±0.39	7.36±0.24	7.09±0.03*	6.18±0.08	6.16±0.07	30.01
	600b	10.74±0.35	7.82±0.25	5.33±0.17	5.08±0.05	4.46±0.42	4.06±0.04*	4.04±0.04*	46.13
N. hex	300a	10.61±0.28	8.53±0.41	7.85±0.19	7.35±0.10	6.89±0.36	6.14±0.14	6.13±0.14	30.85
	600b	10.60±0.25	7.70±0.22	5.41±0.25	5.10±0.05	4.89±0.13	4.10±0.08	4.08±0.07	45.62
Ethyla	300a	10.44±0.13	8.82±0.12	8.36±0.35	8.04±0.25	8.02±0.09	7.78±0.31	7.58±0.25	23.92
	600b	10.67±0.27	7.15±0.14	6.15±0.03*	5.51±0.28	5.07±0.06	4.78±0.17	4.76±0.19	42.91
N. but	300b	10.59±0.18	6.73±0.50	6.06±0.09	5.63±0.28	5.21±0.07	4.72±0.29	4.70±0.30	43.48
	600b	10.90±0.16	5.56±0.25	4.11±0.07	4.05±0.04*	3.91±0.11	3.05±0.05	3.04±0.05	74.24
Water fract	300b	10.31±0.07	7.13±0.35	6.93±0.45	6.63±0.41	6.02±0.41	5.28±0.42	5.26±0.41	38.25
	600b	10.65±0.14	6.19±0.14	5.20±0.08	5.08±0.20	4.94±0.04*	4.26±0.15	4.23±0.15	47.47
Pure diclofenac	50	10.66±0.15	8.05±0.36	6.45±0.28	6.14±0.08	6.04±0.08	5.67±0.41	5.61±0.42	37.13

KEY: One-way ANOVA: values expressed as mean± SEM, n=5 in each group*p>0.05 and TV>CV.b=means significant effect than+ctrl, a=means insignificant than+ctrl,*=correspond to p-value>0.05, green= -ctrl, blue and purple=fractions with highest activity than+ctrl.

Table 5: *In vivo* test result for crude extract and fractions of P. *discoides* bark on carrageenan induced paw edema induced in rats

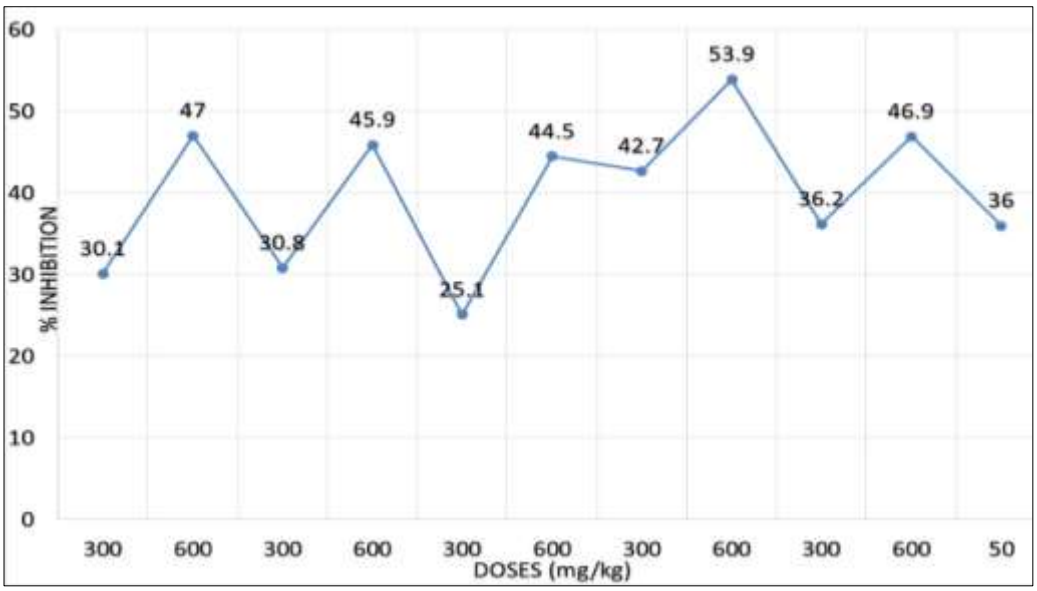
Sample	Dosage mg/kg	0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	24 hrs	% inhibition
Negative control	10 ml NS	10.76±0.13	10.88±0.17	10.89±0.40	11.18±0.45	11.19±0.12	11.31±0.13	11.54±0.14	-
Methanol extract	300a	10.68±0.17	9.09±0.48	7.66±0.33	7.29±0.17	7.11±0.04*	6.20±0.07	6.09±0.04*	30.10
	600b	10.79±0.28	7.56±0.29	5.29±0.09	5.11±0.02*	4.50±0.20	4.09±0.06	4.05±0.03*	47.00
N. hexane fraction	300a	10.65±0.27	8.61±0.3	7.79±0.23	7.33±0.09	6.89±0.35	6.15±0.03*	6.09±0.02*	30.80
	600b	10.58±0.28	7.38±0.10	5.44±0.20	5.10±0.03*	4.89±0.12	4.21±0.06	4.18±0.04*	45.90
Ethyl acetate fraction	300a	10.49±0.16	8.82±0.50	8.14±0.14	8.04±0.09	7.91±0.06	7.46±0.08	7.13±0.04*	25.10
	600b	10.59±0.24	7.15±0.07	6.10±0.07	5.54±0.27	5.18±0.09	4.84±0.05	4.32±0.05	44.50
N. butanol fraction	300b	10.60±0.16	6.61±0.31	6.05±0.06	5.66±0.27	5.21±0.08	5.15±0.02*	5.00±0.14	42.70
	600b	10.94±0.13	5.40±0.24	4.09±0.05	4.04±0.02*	3.96±0.06	3.78±0.04*	3.63±0.07	53.90
Water fraction	300b	10.34±0.06	7.39±0.14	7.09±0.12	6.6±0.30	6.14±0.07	5.90±0.10	5.78±0.05	36.20
	600b	10.63±0.17	6.24±0.15	5.23±0.07	5.11±0.03*	4.85±0.05	4.58±0.04*	4.38±0.05	46.90
Pure diclofenac	50	10.76±0.28	7.94±0.19	6.56±0.29	6.15±0.04*	6.12±0.43	6.09±0.06	6.02±0.04*	36.00

KEY: One- way ANOVA: values expressed as mean ± SEM, n=5 in each group*p>0.05 and TV>C, b=means significant effect than+ctrl, a=means insignificant than+ctrl,*=correspond to p-value>0.05, yellow color=-ctrl, blue and purple=fraction with highest activity than+ctrl



KEY: 30.01-46.13 (methanol extract), 30.85-45.62 (hexane fraction), 23.92-42.91(ethyl acetate fraction), 43.48-74.24 (butanol fraction), 38.25-47.47(water fraction), 37.13 (diclofenac)

Fig 2: Graph of % inhibition /doses for egg albumen induce



KEY.30.1-47 (Methanol extract), 30.8-45.9 (hexane fraction), 25.1-44.5 (ethyl acetate fraction), 42.7-53.9 (butanol fraction), 36.2-46.9 (water fraction), 36 (diclofenac).

Fig 3: Graph of % inhibition/doses for carrageenan induce

Discussion

Phyllanthus discoides bark is one of indigenous traditional listed herbal plant of medicinal important, study from this work reviewed its wide spectrum of anti-inflammatory activities as compared to diclofenac pure sample (NSAID) whose mechanism of action is by inhibition of cycloxygenasa-1(COX-1) in arachidonic acid pathway. The result in table 1 showed that ethyl acetate gave the highest percentage yield followed by the aqueous fraction. This is in line with the previous study of *P. discoides* as stated by (Brijesh, Sunil, & Madhusudanan, K. P, 2020) [5].

Table 1 also showed that *P. discoideus* have good yield in methanol solvent using cold maceration method with ethyl acetate fraction giving the highest yield value of all solvent options used for fractionation (n-hexane, ethyl acetate, n-butanol and water fraction).This illustrate that the phytochemical constituent present are intermediate in solubility between polar and nonpolar solvent with n-hexane fraction having list % yield in line with previous studies.

Phytochemical screening test of both the crude and fractions gave different secondary metabolites; triterpenoids, flavonoids, alkaloids, tannins, saponin, steroids, phenolic compounds and carbohydrate as shown in Table 2. Previous studies have shown that *Phyllanthus* deciduous contains those secondary metabolites (Harborne J.B, 2010) [9]. The result of this present study showed that flavonoids, alkaloids and phenolic are dominant compounds as shown in table 2. The result of the acute toxicity studies in Table 3 showed no obvious toxicity or death at 5000 mg/kg showing that the LD_{50} of the leaves of *Phyllanthus discoideus* is greater than 5000 mg/kg.

The therapeutic effect of *P. discoideus* crude extract and fractions as anti-inflammatory agents were able to alleviate inflammatory condition in rat using curative models (Carrageenan and egg albumen) study.

Analysed result as shown in table 4 and 5, and figure 2 and 3 proved n-butanol fraction to have the highest anti-inflammatory efficacy, potency and better curative effect on both two model studied (egg albumen and carrageenan induced effect).

In figure 2 and 3, studies on dose response relationship showed that increase in dose will respond to increase in therapeutic response in perfect linear progression until maximum plasma concentration attained which begins to show little inhibition capacity. From the dose studied, the effect of both 300 mg and 600 mg doses against percentage inhibition zone yielded positive response with more effect towards 600 mg/kg. Analysis of duration of action, peak time and time to achieve CMAX therapeutic profile were obtained between 1-3 hours with the peak effect at 2hrs while decline in therapeutic effect starts from 4hrs down as shown in table 4 and 5.

A comparative study of the anti-inflammatory effect of n-butanol and aqueous fractions of *P. discoideus* with positive control (diclofenac) as contained in table 4 and 5 reviewed *that P. discoideus* gave a better anti-inflammatory effect. Previous research works and review reports have reported most *Phyllanthus* species to have promising anti-inflammatory activities (A Kindele, *et al*, 2015) [2]. However, the result of this present study on the stem bark of *P. discoideus* gave highest anti-inflammatory effects compared to other previous finding in phyllanthaceae family.

Conclusion

Evidence from the results of this study proves that the stem bark of *Phyllanthus discoideus* has a good promising anti-inflammatory effect with n-butanol fraction having the highest profile of anti-inflammatory activity which is a proof that the phytoconstituents responsible for the said activity resides within the polar region. It is also evident from the result that n-butanol fraction has more anti-inflammatory effect than the widely used diclofenac. Therefore, the stem bark of *P. discoideus* will give a therapeutically useful anti-inflammatory drug candidate that can be isolated on further research.

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