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1. **Full title:**
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4. **Abstract:**

Ethnopharmacological relevance: Preparations of *Olax subscorpioidea* have been used traditionally for the management of pains, inflammatory diseases, yellow fever, cancer and rheumatism. Previously, the analgesic activity of its leaf extract have been reported. Furthermore, an analgesic assay guided fractionation showed that the butanol soluble fraction is the most active. However, the mechanism of this activity remains to be elucidated. This present study investigated the possible pharmacological mechanisms

involved in the analgesic activity of the butanol leaf fraction of *Olax subscorpioidea* (BFOS) using the acetic acid induced writhing test in mice.

Materials and Methods: Animals were orally administered distilled water (10 ml/kg), BFOS (1,000 mg/kg) and morphine (10 mg/kg) 60 minutes before *i.p* administration of acetic acid and the resulting writhing were counted for 10 minutes. To establish the possible mechanism(s) of action of BFOS, separate group of animals were pretreated with naloxone (2 mg/kg, *i.p*), prazosin (1 mg/kg, *i.p*), yohimbine (1 mg/kg, *i.p*), propranolol (20 mg/kg, *i.p*), metergoline (2 mg/kg, *i.p*), glibenclamide (5 mg/kg, *i.p*) and l-arginine (50 mg/kg, *i.p*) 15 minutes before BFOS.

Results: BFOS and morphine showed marked analgesic activities ($p<0.001$); the pretreatment of animals with naloxone, metergoline and l-arginine significantly ($p<0.05$ and $p<0.001$) reduced the analgesic activity of BFOS; however, pretreatment with prazosin, yohimbine, propranolol and glinbenclamide showed no effect on its analgesic 0activity.

Conclusion: Results obtained in this study suggest the involvement of opioidergic, serotonergic and nitric oxide-l-arginine pathways in the analgesic effect of butanol leaf fraction of *Olax subscorpioidea*.

5. **Keywords:**
Olax subscorpioidea, analgesic, opioidergic, serotonergic, nitric-oxide and pain mechanism.

1. Introduction

Traditional herbal medicine is still believed to be the most abundant, affordable, reliable, trusted and well-understood form of health care in Africa, as over 80% of its populations use some form of traditional herbal medicine (Awodele et al., 2012); and an impressive number of modern drugs have been isolated from natural sources (Calixto et al., 2009).

Medicinal plants and their products have been used for many centuries to treat different kinds of acute and chronic pains. One of such medicinal plants with wide patronage is *Olax subscorpioidea.* It is an olacaceae family member, widely distributed in Africa tropics with several uses and is commonly known in different African languages as *Ifon* or *Ufon* (Yoruba), *Gwaanon kurmii* or *Gwaanon raafii* (Hausa), *Igbulu, Atu-ogili* or *Osaja* (Igbo), *Ukpakon* (Edo)

and *Ocheja* (Igala). It is used traditionally for the management of pain and related diseases (Odoma et al., 2014).

A good number of plant products with analgesic activity have been documented, but very few of these compounds have reached clinical use due to scant scientific evidence that could explain their mode of action (Bellik et al., 2013). Previously, the analgesic activities of the methanol crude extract of *O. subscorpioidea* and its butanol, hexane and residual aqueous fractions have been reported (Odoma et al., 2014; Odoma et al., 2015). In view of the previous results on the analgesic activity guided fractionation which showed that the butanol soluble fraction is the most active (Odoma et al., 2015); the purpose of the present work was to investigate the possible mechanism for the analgesic action of the butanol fraction. This will promote the discovery of promising targets for the development of new drugs to treat chronic pain. Thus we investigated the participation of opioidergic, adrenergic, serotonergic, potassium ATP and nitric oxide-l-arginine pathways in the analgesic activities of the butanol leaf fraction of *O. subscorpioidea* (BFOS) on acetic acid induced writhing test in mice.

2. MATERIALS AND METHODS

2.1 Collection and identification of the plant

The leaves of *O. subscorpioidea* were collected from a farm in the premises of College of Health Sciences, Kogi State University, Anyigba, Kogi State, Nigeria, in March 2013 and identified by Dr. Emmanuel I. Aigbokhan, a taxonimist at the Department of Biological Sciences, Faculty of Natural Sciences, Kogi State University, Anyigba, Kogi, Nigeria where a voucher number (KSUH-277-2013-01) was deposited for future references.

2.2 Extraction and fractionation

The method previously described by Kupchan et al. (1973) was adopted for the extraction and fractionation. The leaves of *O. subscorpioidea* were shade dried until constant weight was obtained and then grounded into powder with the aid of a mortar and pestle. One kilo gram (1kg) of the powdered leaf material was extracted exhaustively with aqueous-methanol (80% methanol in water) using continuous soxhlet apparatus for 48 hr. The solvent was removed by placing the extract on water bath set at 50°C. One hundred grams (100 g) of the crude methanol extract was dissolved in distilled water and further fractionated into hexane, ethyl-acetate, butanol and residual aqueous fractions. The butanol was removed by placing the fraction on water bath set at 50°C. The fraction was sealed in a bottle container and stored in a desiccator prior to use. Subsequently, it was referred to as "Butanol Leaf Fraction of *Olax subscorpioidea"* (BFOS). Solutions of BFOS were prepared freshly with distilled water for each study.

2.3 Phytochemical screening

The phytochemical screening for the presence of alkaloids, flavonoids, saponins, cardiac glycosides, tannins, anthraquinones, carbohydrates, steroids and triterpenoids in BFOS was done as previously described (Evans, 2009).

2.4 Drugs and chemicals

Glacial acetic acid (May and Baker limited, Dagenham, England), l-arginine, naloxone hydrochloride, metergoline, prazosin, glibenclamide, yohimbine hydrochloride, propranolol hydrochloride (Abcam Biochemicals Plc, Cambridge, UK) and morphine sulphate (Martindale Pharmaceuticals, U.K) were used in the study.

2.5 Animals

Swiss albino mice (18-23g) of either sex were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. The

animals were maintained under standard environmental conditions and fed with standard rodent pellet diet (Vital feed, Jos, Nigeria) and water *ad libitum.* The experiments were approved by the Ethical Committee of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria (protocol number: DAC/IW-OT/137/14) and were carried out in accordance with the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health (Publication No. 80-23, revised 1996).

2.6 Acute toxicity studies

The oral acute toxicity studies of BFOS in mice were conducted according to the method of Lorke (1983) in two phases. In the first phase, 3 groups of 3 mice each were administered 10, 100 and 1,000 mg/kg BFOS. The animals were observed for signs of toxicity and death for the first 4 hours and intermittently for 24 hours. In the second phase, 3 mice were administered 1600, 2900 and 5,000 mg/kg BFOS and were also observed for signs of toxicity and death for the first 4 hours and intermittently for 24 hours. The median lethal dose (LD_{50}) value was determined by calculating the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived.

2.7 Measurement of analgesic activity

The acetic acid-induced writhing test in mice as previously described by Koster et al. (1959) was adopted for the analgesic study. Mice were randomly divided into 3 groups (n=6) and were orally administered distilled water (10 ml/kg), BFOS (1,000 mg/kg) and morphine (10 mg/kg). 60 minutes after oral administration, acetic acid 0.6% v/v (10 ml/kg) was intraperitoneally administered to each mouse. Five minutes after acetic acid injection, mice were placed in observation cage and the number of writhes was counted for each mouse for a period of 10

minutes. A reduction in the number of writhes as compared to the control animals was considered as evidence for the presence of analgesia and expressed as percent inhibition of writhes.

Percentage Inhibition (%) =

$$\frac{\text{Mean No. of writhes (Control)} - \text{Mean No. of writhes (Test)}}{\text{Mean No. of writhing (Control)}} \times 100$$

2.8 Elucidation of the possible mechanism of analgesic action of BFOS

To investigate the possible mechanism of analgesic action of BFOS, randomly selected groups of mice (n=6) were pretreated intraperitoneally with one of the following antagonists or blockers: naloxone (2 mg/kg), prazosin (1 mg/kg), yohimbine (1 mg/kg), propranolol (20 mg/kg), metergoline (2 mg/kg), glibenclamide (5 mg/kg) and l-arginine (50 mg/kg) 15 minutes before oral administration of BFOS (1,000 mg/kg), 2 separate groups were orally administered distilled water (10 ml/kg) and BFOS (1,000 mg/kg) respectively. 60 minutes post administration, acetic acid 0.6% v/v (10 ml/kg) was intraperitoneally administered to each mouse to induce writhing. Pain score was observed and recorded as earlier described.

2.9 Statistical analysis

Values were expressed as Mean ± Standard Error of the Mean (SEM). The data were analyzed by one way analysis of variance (ANOVA) followed by Dunnett or Bonferroni's post hoc test for Multiple Comparison using the graph pad prism (statistical) software. The differences between means were considered significant at $p \leq 0.05$.

3. RESULTS

3.1 Phytochemical screening

The preliminary phytochemical screening of BFOS revealed the presence of saponins, tannins, cardiac glycosides, flavonoids, alkaloids and carbohydrates

3.2 Acute toxicity test

In the acute toxicity test, the oral median lethal dose of BFOS was estimated to be greater than 5,000 mg/kg in mice.

3.3Analgesic activity

The intraperitoneal injection of acetic acid elicited the writhing syndrome in control mice with 33.00 ± 2.31 writhes counted in 10 minutes. BFOS and morphine produced significant (p<0.001) reductions in the number of writhes with peak effect of 59.09% and 79.30% inhibition respectively (Figure 1).

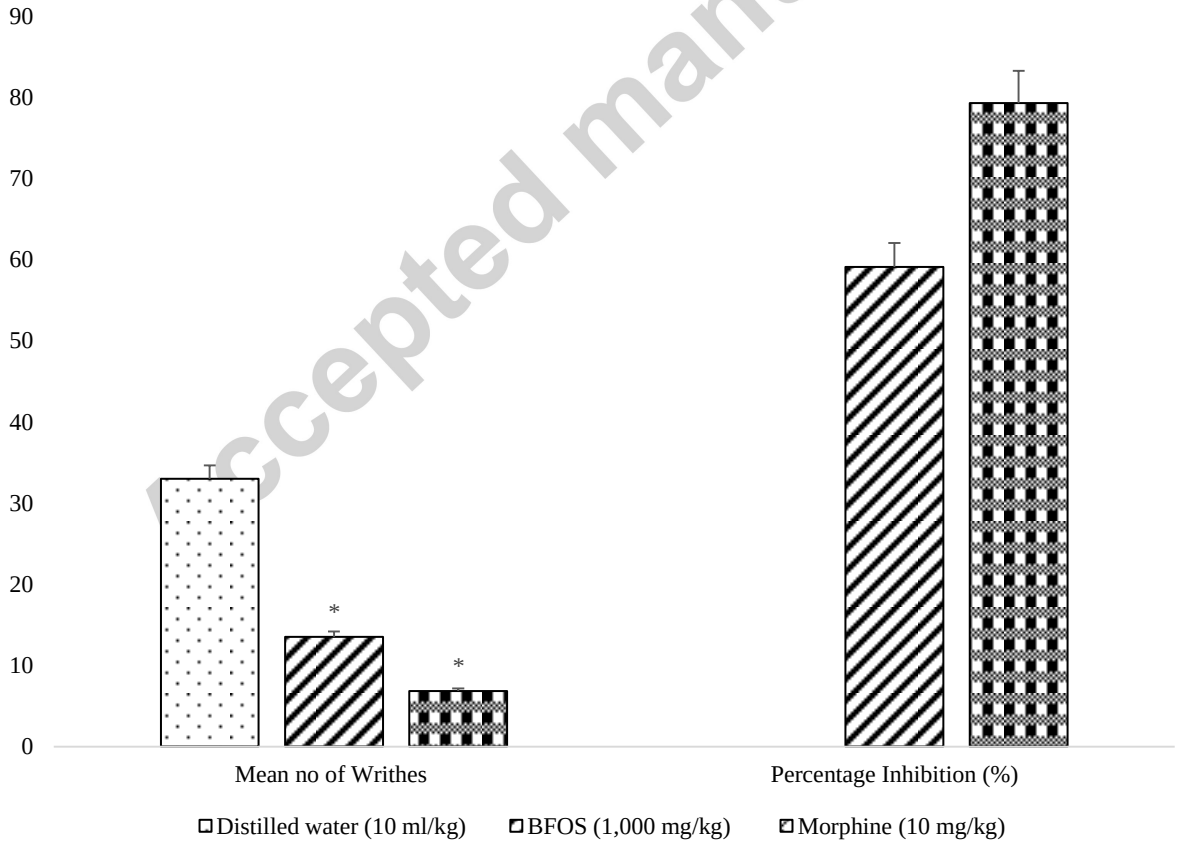

Figure 1: Effects of butanol leaf fraction of *Olax subscorpioidea* on acetic acid induced writhing in mice. Values represent mean ± SEM, * p<0.001 versus control (one-way ANOVA followed by Dunnett's post hoc test), BFOS= Butanol leaf fraction of *O. subscorpioidea*, n=6.

3.4 Mechanistic study

The pretreatment of animals with naloxone, prazosin, yohimbine, propranolol or glibenclamide each did not significantly decrease or increase the number of writhes produced by BFOS. However, the pretreatment of animals with metergoline and l-arginine significantly (p<0.05 and p<0.01 respectively) increased the number of writhing activity of BFOS (Figure 2).

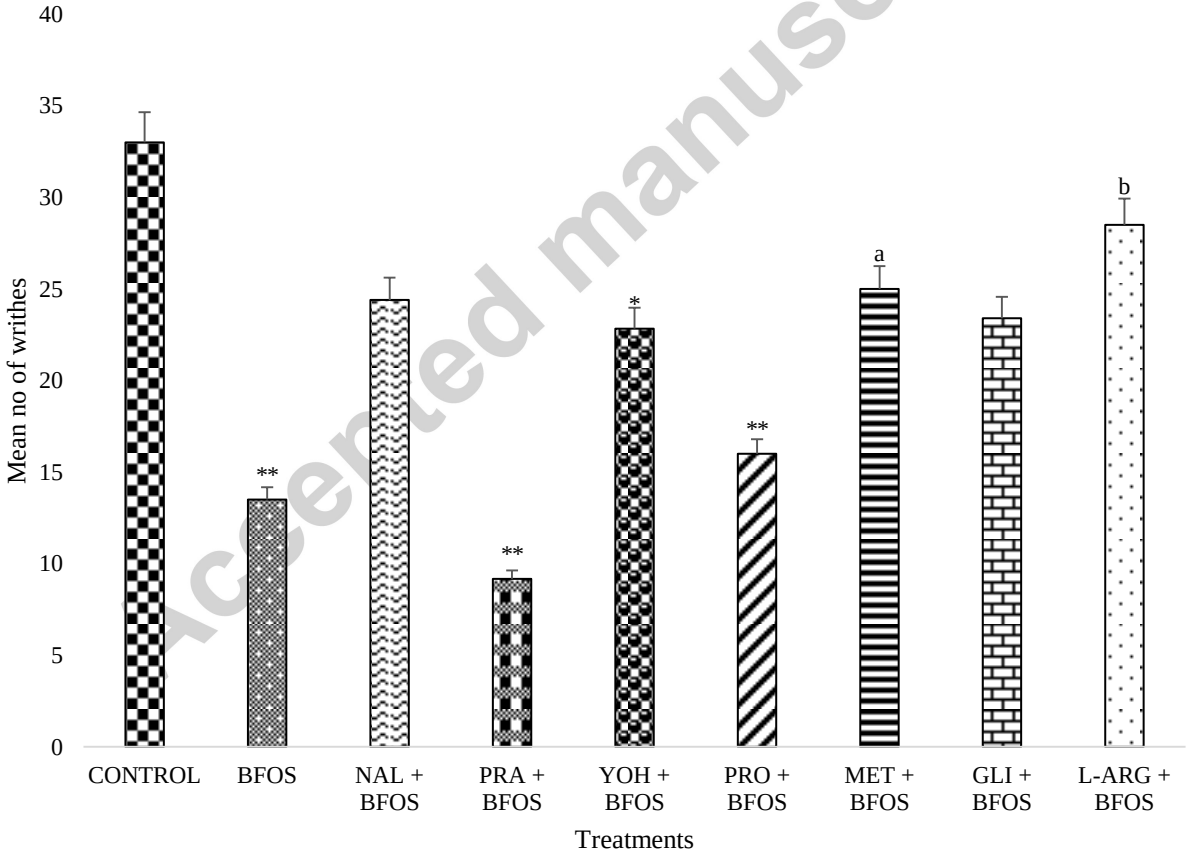

Figure 2: Effect of different receptor blockers on analgesic activity of butanol fraction of *Olax subscorpioidea* on acetic acid-induced writhing test in mice. Values presented as Mean ± SEM; * p<0.05, ** p<0.001 versus Control, [a] p<0.05, [b] p<0.001 versus BFOS (one-way ANOVA followed by Bonferroni's post hoc test for Multiple Comparison), BFOS=Butanol fraction of *O.*

subscorpioidea, NAL=naloxone, PRA=prazosin, YOH=yohimbine, PRO=propranolol, MET=metergoline, GLI=glibenclamide, L-ARG=l-arginine, n=6.

4. Discussion

The present study aimed at elucidating the pharmacological mechanism by which the butanol leaf fraction of *Olax subscorpioidea* (BFOS) exerts its analgesic activity in acetic acid induced pain model. Acetic acid-induced writhing test is a classical chemical/inflammatory pain model widely used to access novel analgesic agents and their mechanism of analgesic actions because it is an easy to learn, fast and replicable model that requires no special equipment (Pavao-de-Souza, et al., 2012).

Acetic acid stimulates pain peripherally and centrally (Mishra et al., 2011; Pavao-de-Souza, et al., 2012). It causes pain by liberating endogenous substances such as serotonin histamine, prostaglandins (PGs), bradykinins and substance P, endings (Mishra et al., 2011) and stimulates central pain by the activation of mitogen-activated protein (MAP) kinases and microglia in the spinal cord (Pavao-de-Souza et al., 2012; Zhang et al., 2011). It also modulates central pain via a number of complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems (Mishra et al., 2011).

Phytochemical analysis of BFOS revealed the presence of some phytochemicals such as alkaloids, tannins, flavonoids, cardiac glycosides, carbohydrates and saponins. Wide ranges of phytochemicals have been reported to be responsible for analgesic activities of medicinal plants; such phytochemicals include flavonoids, saponins, alkaloids and tannins (Anilkumar, 2010; Bellik et al., 2013; Wang et al., 2008). Therefore, the observed pharmacological activities of BFOS may be due to the presence of one or more of the reported phytochemicals.

The oral administration of BFOS up to 5,000 mg/kg in mice caused no death and also no physical sign of toxicity was observed. These suggest that BFOS may be relatively safe (Loomis and Hayes, 1996; Lorke, 1983; Matsumura, 1975) when administered orally.

The results of the present study provide evidence supporting the involvement of the serotoninergic system in the analgesic effect of BFOS as revealed by the finding that pretreatment of animals with metergoline, a serotonin receptor antagonist; significantly reversed BFOS analgesic activities. The serotonergic systems comprise one of the major components of descending pain inhibitory pathways (Dogrul and Seyrek, 2006; Fields et al., 2006; Yoshimura and Furue, 2006). Serotonin released from platelets is able to activate nociceptors (Lang et al. 1990). Studies have suggested pronociceptive effects for serotonin (Pickering et al., 2003; Zeitz et al., 2002) and that the antinociceptive activities of various analgesics depend on integrity of descending serononergic system (Dogrul and Seyrek, 2006; Millan, 2002). Another interesting result of the present study was the demonstration that the L-arginine-nitric oxide pathway is likely to be involved in the activity of BFOS. This conclusion derives from the fact that the pretreatment of mice with the substrate of nitric-oxide synthase, l-arginine, largely reversed the analgesia caused by BFOS when assessed in the acetic acid-induced writhing test. Nitric-oxide (NO) is produced from l-arginine by a chemical reaction catalyzed by the enzyme inducible nitric oxide synthase (iNOS) in living systems. After stimulation with bacterial lipopolysaccharide (LPS), many cells including macrophages express the iNOS which is responsible for the production of large amount of NO (Makchuchit et al., 2010). NO-l-arginine pathway has been shown to participate in thermal inflammatory hyperalgesia and the nociceptive transmission of neuropathic pain; it is said to play a critical role in the glutamate and N-methyl-D-aspartate (NMDA) mediated nociceptive response (Gultekin and Ahmedov, 2006). Over

production of NO have also been reported in a number of clinical disorders including convulsions, pain and schizophrenia (Kiran and Srikanth, 2014). The analgesic activity of BFOS may not involve the opioidergic, adrenergic and K_{ATP} channel pathways. These notions are because the pretreatment of mice with naloxone (a nonselective opioid receptor antagonist), prazosin (a α_1-adrenoceptor antagonist), yohimbine (a α_2-adrenoceptor antagonist), propranolol (a β-adrenoceptor antagonist) and glibenclamide (a potassium channel blocker) each failed to reverse the analgesic activity of BFOS.

5. Conclusion

In conclusion, the precise mechanisms through which the BFOS exerts its analgesic action are not completely understood but seem to involve an interaction with serotonergic and nitric oxide-l-arginine pathways.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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