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Serum levels of oxytocin in pregnancy, parturition and postpartum for Nigerian females in Zaria, Nigeria

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Oxytocin is a hormone involved with adjustment of pregnancy, the process of delivery, breastfeeding, social recognition and bonding. This study aimed at determining the serum levels of oxytocin in pregnancy, during labour, and in the puerperium for Nigerian females in Zaria. It was a cross-sectional study of one hundred and twenty women aged 18 to 45 years from four hospitals in Zaria. The women were grouped into four groups comprising non-pregnant women (control), pregnant women (first, second, and third trimester), women in labour and in their first week after delivery. Questionnaires were administered to the women and their blood samples collected via venipuncture between 09.00 and 13.00 h. After centrifuging the blood samples, the sera were analyzed with human oxytocin ELISA kits at the Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Shika. Results were presented as frequencies, percentages and mean \pm standard deviation (SD) while data was analyzed using one-way analysis of variance (ANOVA), Tukey post-hoc test and the reference range (defined as 95% confidence limits) was determined. A statistical significance of $p < 0.05$ was selected. Serum oxytocin levels (non-pregnant controls: 82.80 ± 23.68 pg/ml; 95% Confidence Interval (CI): 73.02 to 92.58 pg/ml) rose progressively with advancing gestation (first trimester: 167.56 ± 24.17 pg/ml, 95% CI: 148.98 to 186.13 pg/ml; second trimester: 377 ± 53.113 pg/ml, 95% CI 358.50 to 396.17 pg/ml), but there were no significant differences in serum oxytocin concentration for women in their third trimester (499.06 ± 42.06 pg/ml; 95% CI: 483.64 to 514.49 pg/ml) as compared to women during labour (525.0 ± 35.98 pg/ml; 95% CI: 497.35 to 552.65 pg/ml) and the puerperium (spontaneous vaginal delivery: 532.25 ± 29.93 pg/ml; 95% CI: 507.23 – 557.27 pg/ml; caesarean section: 502.40 ± 42.34 pg/ml; 95% CI: 449.83 to 554.97 pg/ml), $p > 0.05$. Maternal serum oxytocin levels at spontaneous vaginal delivery (532.25 ± 29.93 pg/ml; 95% CI: 507.23 to 557.27 pg/ml) were higher than those at caesarean section (502.40 ± 42.34 pg/ml; 95% CI: 449.83 to 554.97 pg/ml) though not statistically significant ($p > 0.05$). In conclusion, it appears that maternal oxytocin levels in Nigerian females though following the pattern in other studies had higher values.

Key words: Enzyme immunoassay, lactation, pregnancy hormone, reference range, serum oxytocin, Zaria.

INTRODUCTION

The neurohormone oxytocin (OTC) is known for its involvement in the process of delivery, reproductive behavior and its physiological role in the onset and

maintenance of lactation (Thackare et al., 2006; Zamiri et al., 2001; Argiolas and Gessa, 1991). Oxytocin has also been linked to metabolic, analgesic, anxiolytic and health-

promoting cardiovascular effects (Grewen et al., 2008; Ring et al., 2006; Holst et al., 2002; Petersson et al., 1999).

Maternal OTC concentrations have been associated with autism related disorders (Nyffeler et al., 2014; Bartz and Hollander, 2008). Lower blood oxytocin levels in autistic children were associated with higher social deficits (Parker et al., 2014; Andariet al., 2010; Modahl et al., 1998). The two comparison groups of the study by Parker et al. (2014), consisted of children with autistic siblings and those without autistic siblings. All the groups demonstrated social skills correlating with their oxytocin levels. Variants of the oxytocin receptor also correlated with social ability and are suggested to serve as predictors of attachment in human infants (Chen et al., 2011).

Beyond these roles, OTC plays a major role in adjustment to pregnancy, maternal behavior and bonding (Wittig et al., 2014; Bartz et al., 2010; Feldman et al., 2007; Curley and Keyerne, 2005; Bartz and Hollander, 2006; Kendrick, 2000). Higher postpartum maternal-fetal attachment scores were observed in women with an OTC rise between the first and third trimester compared to women with stable or decreasing patterns of OTC (Levine et al., 2007). Another study revealed the development of postpartum depression in women having lower oxytocin concentration in mid-pregnancy (Skrundz et al., 2011). Variants of the oxytocin receptor have been associated with depressive symptomatology (Saphire-Bernstein et al., 2011).

OTC also plays a role in the social behavior of humans (Meyer-Lindenberg et al., 2011; Ebstein et al., 2010). In other subjects, OTC was observed to promote interpersonal relationships and enhance feelings of love and trust (Krueger et al., 2012). Evidence suggests that more intimate, positive social affiliations may protect and prolong good health (Kiecolt-Glaser and Newton, 2001; Berkman, 1995). Intra-nasally administered OTC was also found to increase adult attention to the eye region of faces and to facilitate their recognition of positive social words (Unkelbach et al., 2008; Guastella et al., 2008a).

In medical practice, measurement of hormone concentrations is important for the evaluation and treatment of diseases. Ethnic variations in hormone reference ranges exist (Sachidhanandam et al., 2010; Pinheiro et al., 2005; Potischman et al., 2005). Considering the relevance of oxytocin concentration to several physiological parameters and social behaviors, this study aimed at determining the reference range of serum oxytocin for Nigerian women in Zaria. A difference was expected in the reference ranges of serum oxytocin

of Nigerian women in pregnancy, labour and the puerperium.

METHODOLOGY

Study site

The study was conducted among women in Zaria, Northern Nigeria. Zaria is a town located within latitude 11°3'N and longitude 7° 42'E. The town is comprised of 2 local government areas with an altitude of 610 m, an annual rainfall of 1056.6 mm and a mean annual temperature of 27°C (Mortimore, 1970).

Study design

The study was a cross-sectional, multicenter study. Women were recruited from antenatal clinics, delivery rooms and lying-in wards of five hospitals in Zaria. They consisted of Primary Health Centre, Samaru; Comprehensive Health Centre, SabonGari; Salama Hospital and Maternity, Kwangila; St Luke's Hospital, Wusasa; and Ahmadu Bello University Health Centre, Samaru, Zaria. While the control subjects consisted of individuals from Ahmadu Bello University (students and staff). Approval for the study was obtained from the Ethical Committee on Human Study of the Kaduna State, Ministry of Health. All participants provided informed consent.

Participants

A total of one hundred and twenty healthy female subjects participated in the study. Twenty-five subjects served as controls for the study. They were non-pregnant, non-lactating women in their reproductive age who were not on hormonal contraceptives. A total of ninety-five women were either pregnant, in labour or in the puerperium. All pregnancies were dated to the last menstrual period. Nine women were in their first trimester, 33 women were in their second trimester while thirty-one women were in their third trimester. Nine women were in labour and 13 were women in puerperium; eight of which had spontaneous vaginal delivery (SVD) and five had a caesarean section (CS). Subjects were screened for the following exclusion criteria: (1) medical complications, such as diabetes mellitus or hypertension; (2) smoking.

Sociodemographic variables

A questionnaire was administered to all participants to obtain bio-socio-demographic data about age, educational level, marital status, parity and ethnicity from self-report.

Weight, height and body mass index (BMI) assessment

The weights of the subjects were measured while wearing light clothing to the nearest 0.2 kg with a calibrated weighing scale. Height (without shoes and head attire) was measured to the nearest 0.5 cm with a stadiometer. The BMI was calculated as weight (kg)/height (m²) (Guyton and Hall, 2006).

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Blood pressure measurement

The subjects were seated for 5 min with legs uncrossed, while a mercury sphygmomanometer (Acosson, A. C. Cossor & Son (surgical) LTD, London) and a 3M Littmann Classic II S.E. Stethoscope (3M Health Care, U.S.A) was used to determine the blood pressure by auscultation (Pickering et al., 2005).

Blood sample collection

Participants were instructed about the procedure on arrival at the health facility, they rested quietly for a minimum duration of 30 min to 1 h. Other parameters were also obtained on the same day (some participants had to proceed for their antenatal visits thereafter). The blood samples were taken between 09.00 and 12.00 h. Participants were seated on an examining couch while 5 ml of venous blood was collected at the cubital fossa using a 5 ml syringe and a 23G needle. The sample were then transferred to clean, plain and dry bottles which were kept cold and centrifuged using a bench centrifuge at 1,000 × g for 15. The serum was pipetted into plain bottles and frozen at -8°C until analysis.

Blood sample analysis

Samples were analyzed at the Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Shika, Zaria. Using Human Oxytocin, OT ELISA kit (EIAab). The principle of the test employed the quantitative sandwich enzyme immunoassay technique. Serum concentration of OTC was determined according to the protocol of the commercial kits and by measuring the detectable color intensity using a Microwell reader at 450 nm (Tietz, 1995). Assay sensitivity was <11.7 pg/ml with a detectable range of 15.6 to 1000 pg/ml specificity was for recombinant and human oxytocin with no significant cross-reactivity (Tietz et al., 2000).

Data analysis

Results were presented as frequencies, percentages and mean ± standard deviation (SD). Data was analyzed using one-way analysis of variance using SPSS version 22, followed by a Tukey Post-hoc test and the reference range (defined as 95% confidence limits) was determined as arithmetic mean ± 2 SD. Results were considered statistically significant with $p < 0.05$.

RESULTS

The following result is for 120 subjects comprising of non-pregnant controls, women in their first, second, and third trimesters, women in labour and the puerperium. The data presented includes the socio-demographic details, the anthropometric parameters and the serum oxytocin (central 95 percentile) concentration of apparently healthy Nigerian women in Zaria (Tables 1, 2 and Figure 1). Women in the age range of 21 to 25 years constituted the highest proportion of the study subjects. A large proportion of the subjects were Hausa women (49%) while all the women had some form of education with the tertiary education group constituting the highest proportion (38%). Most of the subjects were housewives (35%) while the least were artisans (5%).

There was a significant difference in weight of the pregnant women and women in labour as compared to the non-pregnant control ($p < 0.05$). There was no significant difference in the height and BMI of the women across all groups. Mean systolic blood pressure, diastolic blood pressure and mean arterial blood pressure was significantly increased in women in labour and the puerperium as compared to the non-pregnant control ($p < 0.05$). There was however no significant difference in the blood pressure parameters of pregnant women as compared to the non-pregnant control ($p > 0.05$). There was no significant difference in the age of the women across all groups ($p > 0.05$). There was a progressive increase in mean serum oxytocin level across the trimesters of pregnancy into the puerperium ($p < 0.05$). However, this increase was not significant between the 3rd trimester, labour and postpartum groups ($p > 0.05$).

DISCUSSION

The range of OTC concentration found in our sample of participants, is in line with the findings of Prevost et al. (2014). In their study, they had a range of 32.3 to 2297.6pg/ml. In our study, there was a large inter-individual differences in OTC levels (minimum = 55 pg/ml; maximum = 591 pg/ml respectively). However, other studies have observed a much lower value for peripheral OTC levels in women (Bick and Dozier, 2010; Grewen et al., 2010). The pulsatile release of OTC, laboratory variation in assay methods and variation in sample processing could explain the variations from one study to another.

In line with our hypothesis, it could be shown that OTC concentration showed fluctuations at different stages of gestation and the postpartum period. An observed increase in OTC concentration with increasing trimester was demonstrated (Figure 1). Our findings are in agreement with studies by Feldman et al. (2007), Stock et al. (1991), and deGeest et al. (1985). The prepartum elevated levels of OTC is contributed by maternal pituitary production, maternal uterine sources and fetal pituitary production. On a molecular level, the amount of freely available oxytocin measured by the antibody-based assay might vary due to the amount of albumin circulating (Abduljalil et al., 2012). As albumin (a protein likely to bind oxytocin) levels decrease as pregnancy progresses more, oxytocin becomes available for the assay. This mechanism could explain how oxytocin levels rise during pregnancy. The increase in oxytocin level is in preparation for delivery sequelae to which the later stage of pregnancy is also characterized by an increase in the number of oxytocin receptors (Maggi et al., 1990). In the study by Prevost et al. (2014), women who were pregnant with their first child had higher oxytocin levels in the third trimester and showed larger increases in

Table 1. The sociodemographic parameters of the participants.

Variable	Groups				Totals
	Non-pregnant (n=25)	Pregnant (n=73)	Labour (n=9)	Puerperium (n=13)	
	Frequency (%)	Frequency (%)	Frequency (%)	Frequency (%)	
Age group (years)					
<20	5 (20)	19 (76)	0 (0)	1 (4)	25
21-25	10 (27)	22 (59.5)	1 (2.7)	4 (10.8)	37
26-30	4 (13.8)	22 (79.9)	2 (6.9)	1 (3.4)	29
>31	6 (20.7)	10 (34.5)	6 (20.7)	7 (24.1)	29
Ethnicity					
Hausa	2 (3.4)	43 (72.9)	5 (8.5)	9 (15.3)	59
Igbo	3 (30)	5 (50)	2 (20)	0 (0)	10
Yoruba	9 (52.9)	7 (41.2)	0 (0)	1 (5.9)	17
Others	11 (32.4)	18 (52.9)	2 (5.9)	3 (8.8)	34
Educational Status					
Qur'an	0 (0)	19 (76)	3 (12)	3 (12)	25
Primary	0 (0)	3 (37.5)	2 (25)	3 (37.5)	8
Secondary	3 (7.5)	28 (70)	4 (10)	5 (12.5)	40
Tertiary	22 (47.8)	23 (50)	0 (0)	1 (2.2)	46
Nil	-	-	-	-	-
Occupation					
Civil Servant	8 (53.3)	7 (46.7)	0 (0)	0 (0)	15
Trader	0 (0)	17 (73.9)	2 (8.7)	4 (17.4)	23
Student	17 (50)	16 (47.1)	0 (0)	1 (2.9)	34
House Wife	0 (0)	30 (71.4)	5 (11.9)	7 (16.7)	42
Artisan	0 (0)	3 (50)	2 (33.3)	1 (16.7)	6

Table 2. The mean age, anthropometric measurement and blood pressure of the participants.

Parameter (mean±SD)	Non-pregnant women (n=25)	Pregnant women (n=73)	Women in labour (n=9)	Women in puerperium (n=13)
Age (years)	25.53±5.14	25.30±5.14	32.78±5.12	30.00±6.65
Height(m)	162.69±7.96	163.65±11.28	165.5±1.41	161.30±2.14
Weight(kg)	58.04±10.14	64.08±18.08*	78.38±3.11*	65.45±5.26
BMI (kg/m ²)	21.87±4.41	24.25±5.58	28.25±0.07	25.12±2.14
Systolic B.P(mmHg)	112.36±11.26	109.30±10.19	135.71±19.88*	131.82±19.40*
Diastolic B.P(mmHg)	76.20±9.05	71.69±9.14	87.14±11.13*	90.0±13.42*
Mean Arterial B.P (mmHg)	88.25±8.82	84.23±8.62	103.33±13.19*	103.94±15.26*

B.P: Blood pressure; BMI: body mass index. *p<0.05.

oxytocin levels from the first to the third trimester as compared to mothers who already had one or more children. The rise in prepartum OTC with increasing weeks of gestation is also associated with higher postpartum maternal-fetal attachment scores (Levine et

al., 2007). Plasma OTC concentrations during pregnancy, have been found to be positively associated with a set of maternal bonding behaviors. Behaviors are like positive effect and gaze in interactions, as well as cognitive attachment representations towards the newborn in the

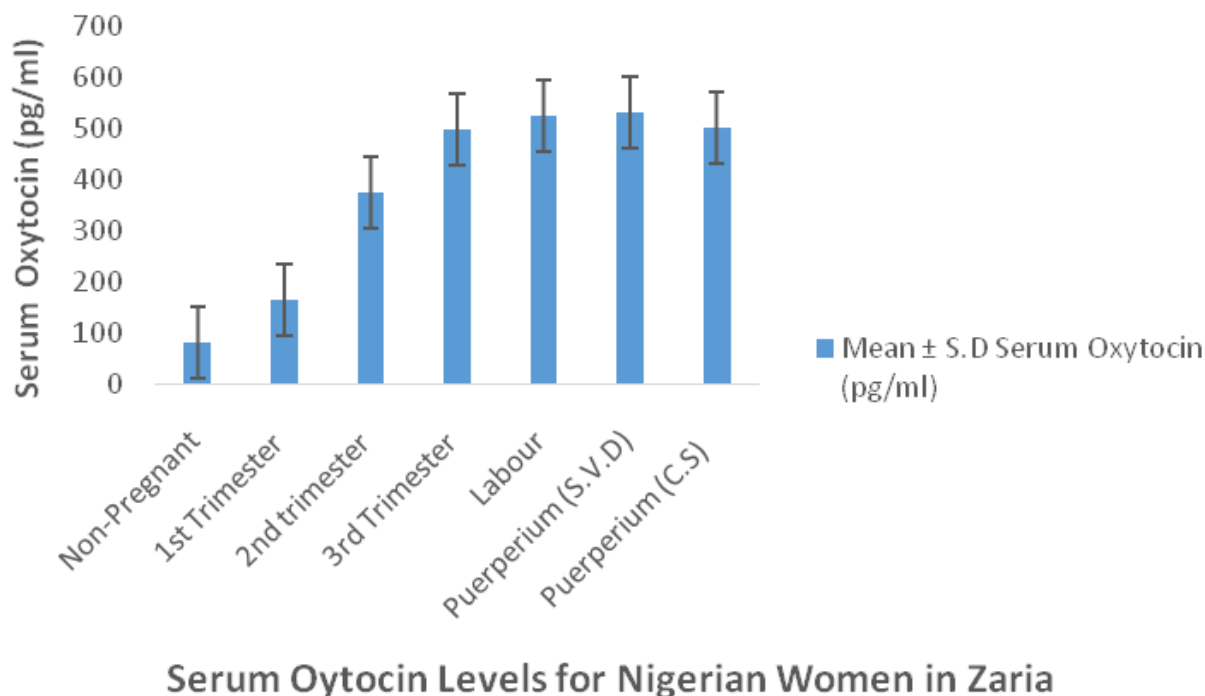


Figure 1. Serum oxytocin levels in non-pregnant, pregnant, in labour and postpartum Nigerian women in Zaria. SVD: Spontaneous vaginal delivery; CS: caesarean section.

early postpartum period (Feldman et al., 2007). While lower OTC levels in pregnancy was found to be associated with a risk for the development of postpartum depression (PPD) (Skrundz et al., 2011). Their study findings suggest that prepartum oxytocin levels could be used to identify women at risk for PPD. The mechanisms behind the observed association between OTC and PPD symptoms remain to be elucidated. Correspondingly, animal studies report deficits in social memory, decreased vocalization and increased aggressiveness in oxytocin knockout mice, while oxytocin knockout rats exhibited deficits in maternal behavior (Winslow and Insel, 2002).

Group comparisons revealed that though maternal OTC levels rose progressively with advancing gestation; there was however no significant difference in the concentration of OTC among women in their third trimester, labour and postpartum (Figure 1, $p > 0.05$). The present findings are also in agreement with the human study by Kuwabara et al. (1987). Their study revealed a lack of significant difference in OTC concentration around the onset of labour. There is however a 100 fold increase in oxytocin receptor concentrations during pregnancy. This accounts for the increased sensitivity of the myometrium during the second half of pregnancy which peaks during early labour (Fuchs et al., 1982). This effect is reversed eventually as shown by the observed

decrease in oxytocin receptors in the postpartum uterus in rats (Soloff et al., 1979).

Maternal oxytocin levels at spontaneous delivery were higher than those at Caesarean section (Figure 1). This was however not significant ($p > 0.05$). This is at odds with a previous report, where significantly higher maternal oxytocin concentration was observed in women that had spontaneous delivery as compared to women that had caesarean section delivery (Kuwabara et al., 1987). Our finding might be due to our subjects comprising of both elective and emergency caesarean section cases.

The findings of this study need to be confirmed in future studies by longitudinal studies assessing OTC over the course of pregnancy, labour and puerperium. Single sample per subject was assayed more than one sample per assessment giving a more accurate measure. The sample consisted predominantly of women of Hausa origin. Consequently, studies need to be replicated with a more heterogeneous population of Nigerian women. Studies involving the effect of peripheral OTC on social behavior of children and adult mothers though elucidated in other populations (Caucasian and African-American samples) should be explored in a Nigerian sample. The use of women in the first week of puerperium could be extended to include further weeks postpartum.

In summary, the findings of this study suggest that serum OTC concentration progressively increase with

increasing weeks of gestation, does not significantly increase during labour and the early postpartum period and the reference ranges for maternal oxytocin levels in Nigerian females, though following the pattern in other studies had higher values. Does this suggest higher maternal-infant attachment scores in Nigerian women? Is a question to be explored in further studies.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vitro* anthelmintic activity of *Allium sativum*, *Allium cepa* and *Jatropha curcas* against *Toxocara canis* and *Ancylostoma caninum

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Prevalence of animal diseases is one of the major livestock production constraints in Kenya with high impacts on livelihoods due to related economic losses affecting food security in the country. The use of synthetic drugs for disease management has challenges. This makes the use of medicinal plants for treatment a rational alternative. Ascarids, *Toxocara canis* and *Ancylostoma caninum* are among the most frequently observed helminth parasites in dogs in Kenya. The two parasites are also known to cause helminthiasis in human beings. This study was designed to evaluate the *in vitro* efficacy of ethanol and aqueous extracts from bulbs of *A. sativum* and *A. cepa* and from leaves of *J. curcas* against *T. canis* and *A. caninum* parasites. Six (6) extracts from three (3) plants: *A. cepa*, *A. sativum* and *J. curcas* were selected for *in vitro* anthelmintic screening by measuring ability to inhibit hatching and development of eggs and survival of larvae *in vitro*. The ethanol extracts of *A. cepa* inhibited hatching of 100% of eggs of *A. caninum* between 10,000 and 2,500 ug/ml and 100% of eggs of *T. canis* between 10,000 and 1,250 ug/ml while that of *A. sativum* inhibited hatching of 100% of *A. caninum* eggs between 10,000 and 5,000 ug/ml. However the ethanol extract of *A. sativum* did not have the same effect on the development of *T. canis* eggs at these concentrations. The ethanol extracts of both *A. cepa* and *A. sativum* affected the survival of 100% of *A. caninum* larvae at a concentration of 156 ug/ml and above. The water extracts of the three plants had moderate effects on the eggs and the larvae of both parasites. The results indicate that the ethanol extracts of *A. cepa* and *A. sativum* have anthelmintic properties which should be investigated further to support the ethnoveterinary use of the plants as anthelmintics for control and treatment of worm infestation in dogs.

Key words: *Allium cepa*, *Allium sativum*, Anthelmintic activity, *Ancylostoma caninum*, *Jatropha curcas* *Toxocara canis*.

INTRODUCTION

The common varieties of *Allium cepa* (onion) locally known as kitunguu and *Allium sativum* locally known as kitunguu sumu are widely grown for their culinary uses.

The leaves of *A. sativum* are long, narrow and flat like grass. The bulb (the only part with culinary uses) is of a compound nature, consisting of numerous bulblets,

known technically as 'cloves,' grouped together between the membranous scales and enclosed within a whitish skin, which holds them as in a sack. The flowers of *A. sativum* are placed at the end of a stalk rising direct from the bulb and are whitish, grouped together in a globular head, or umbel, with an enclosing kind of leaf or spathe, and among them are small bulbils (Grieve, 2006). *A. cepa* is a perennial herb which is strong smelling when crushed. Its roots are adventitious and fibrous. It has an underground stem modified into a tunicated bulb consisting of reduced stem and axillary buds surrounded by inner fleshy scale leaves and outer membranous dry scales. Leaves are radical, alternate, sessile, simple cylindrical, hollow green and parallel veined foliage with a fleshy sheathing base arising from the underground stem (Ranjitkar, 2003). The terminal inflorescence develops from the ring-like apical meristem. It has scapes, one to several, generally elongate well above the leaves and range in height from 30 cm to more than 100 cm (Ross, 2001)

Both *A. sativum* and *A. cepa* were sourced from Uthiru Market located approximately 25 km from Nairobi City Center and their bulbs were used for the experiment. *Jatropha curcas* is a monoecious plant with unisexual or occasionally hermaphrodite flowers. It has a gynoeceum with three slender styles which are connate to about two-thirds of their length, dilating to massive bifurcate stigma. It grows to the size of a small tree or a large shrub, which can reach a height of 3 to 5 m. The branches contain latex. It forms five roots from seedlings, one central and four peripheral. A tap root is not usually formed by vegetatively propagated plants. Leaves have 5 to 7 lobes, hypostomatic and stomata are of paracytic (Rubiaceous) type. It has inflorescence each yielding a bunch of approximately 10 or more ovoid fruits. The seeds are black and the seed weight per 1000 is about 727 g (Kumar and Sharma, 2008).

J. curcas was sourced from the outskirts of Ngong Hills in Kajiado County, approximately 30 km South of Nairobi. The leaves were used for the experiment. Ascarids, *Toxocara canis* (dog ascarid worm) and *Ancylostoma caninum* (dog hookworm) are among the most frequently observed helminth parasites in dogs in Kenya (Kagira and Kanyari, 2000). Both *T. canis* and *A. caninum* are also known to cause helminthiasis in human beings (Dickson, 2003; Juergen and Paul, 2003). The adult stage of the ascarid worm lives in the small intestines of the dog from where their eggs are passed into the environment through feces. Over a period of 2 to 6 weeks the eggs develop to the infective third stage larvae (Azam et al., 2012). Humans and dogs are infected by ingesting eggs containing the third stage larvae (Overgaauw, 1997;

Kasai, 1995) or less frequently tissues dormant larvae in tissues of a paratenic hosts. Once ingested the larvae hatch and migrate through blood vessels into soft tissues (Hill et al., 1985). Puppies from infected bitches are born infected through prenatal transmission. *Toxocara canis* infections in humans are usually mild and asymptomatic in two major forms; visceral toxocariasis and ocular toxocariasis. The adult stage of the hookworm is also common in the dog intestines attaching to the linings with hook like teeth and from where they shed eggs to the environment through feces. Animals get infected by ingesting third stage (L3) infective larvae or by the infective larvae attaching and burrowing into the skin from where they migrate to the small intestines, mature, mate and lay eggs. Puppies can also be infected through their nursing mother's milk. Conventional prophylaxis and treatment is based upon routine administration of febantel and pyrantel pamoate to pregnant bitches and puppies. Extracts of *A. Sativum* have shown *in vitro* anthelmintic efficacy against *Fasciola gigantica* (Kumar, 2014), *Schistosoma mansoni* (Mantawy et al., 2012) and *Hemonchus contortus* (Zafar, 2014). Crude ethanolic and aqueous extracts of *A. cepa* bulbs showed strong anthelmintic activity on *Pheretima posthuma* (earthworm), the earthworms were paralysed within 20 min and died within 30 min of exposure (Abhijeet et al., 2012). For *J. curcas*, hexane, ethyl acetate and ethanol extracts obtained from the seeds were found to have *in vitro* efficacy against *Haemonchus contortus* (Monterio et al., 2011) while the aqueous latex of leaves of the plant was found to have similar efficacy against *Pheretima posthuma* (Hitesh et al., 2014). However, none of these extracts is available in the market for use in treatment of helminthiasis because more studies are required to establish the active ingredients, modes of action and safety. This study was designed to evaluate the *in vitro* efficacy of ethanol and aqueous extracts from bulbs of *A. sativum* and *A. cepa* and from leaves of *J. curcas* against *T. canis* and *A. caninum* parasites.

MATERIALS AND METHODS

Experimental animals

Two mongrel male puppies aged between 6 and 10 weeks, with an average weight of 2.2 kg and showing signs of natural strongyle and ascarid infection were purchased from households in Ndumboini village in Kabete. During the experiment the puppies were housed and fed together in dog kennels at the Faculty of Veterinary Medicine of the University of Nairobi. Screening of the puppies for helminth infection was done by microscopic examination of fecal smears from each puppy. Both strongyle and ascarid eggs were observed.

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Plant collection and identification

Both Alliums are commercially grown for culinary uses and were bought from Uthuru Market in Kabete, Nairobi. The *J. curcas* was collected from Ngong Forest in Kajiado County where it grows naturally as a shrub. All the plant materials were identified at the Kenya National Museums Herbarium in Nairobi.

Preparation of extracts

The bulbs of the Alliums and leaves of the *J. curcas* were oven dried separately at 60°C for 48 to 72 h. Each of the dry plant materials was ground to a fine powder, sealed in paper bags and kept in cool dry place. An aqueous extract of the leaves of *J. curcas* was obtained following the method described by Juliana et al. (2014). A total of 900 ml of distilled water was added to 100 g of dry powder and the mixture was heated at 100°C for 15 min. After cooling, the mixture was centrifuged before filtration through a No.1 filter paper (M&N 615 12.5CM) in order to separate the residues. The filtered solution was then freeze dried to obtain a light green colored powder with a recovery rate of 0.94% relative to dry powder.

The ethanol extract of leaves of *J. curcas* was prepared according to Ekundayo and Ekekwe (2013). 1 L of 99.5% ethanol was mixed with 100 g of dried leaf powder and agitated several times for a period of 72 h. The mixture was filtered through Whatman No. 1 filter paper into a clean beaker and then evaporated over a sand bath at 80°C. Drying was completed in an oven at 40°C to prevent the dry extract from sticking to the walls of the container. This yielded a dark green (near black) colored paste with a recovery rate of 2.61%.

Aqueous extracts of both *A. sativum* and *A. cepa* were obtained as described by Mikhail (2009). A total of 100 g of dry powder from ground dry bulbs of each of the Allium was mixed with 900 ml of distilled water and heated in water bath at 100°C for 30 min. After cooling, the mixture was filtered through Whatman No. 1 filter paper and the filtrate was centrifuged before decanting the supernatant into a clean beaker. The extracts were freeze dried. *A. sativum* yielded a yellowish colored crystalline powder with a recovery rate of 18.25% while *A. cepa* yielded a sticky dark tan (dark brown) colored solid with a recovery rate of 16.75%.

Ethanol extracts of Alliums were obtained according to Akintobi et al. (2013). A total of 100 g of each powder was mixed with 400 ml of 99.5% ethanol and agitated several times for a period of 72 h. The mixture was filtered through a Whatman No. 1 filter paper into a clean beaker and then evaporated over a sand bath at 80°C. Drying was completed in an oven at 40°C to prevent the dry extract from sticking to the walls of the container. The *A. sativum* ethanol extract yielded a mid-brown colored paste with a recovery rate of 0.39% while the *A. cepa* ethanol extract yielded a maroon colored paste with a recovery rate of 2.03%.

Determination of effects on hatching

This was done using the Egg Hatch Assay as described by Le Jambre (1976) and used by other researchers (Coles et al. 2006) with minor modifications.

Determination of effects on survival of larvae (Larval Motility Assay)

This observation was only made on larvae of *A. caninum*. This is because they hatch into free larvae unlike those of *T. canis* which hatch within the egg shells making it difficult to observe their movements. Survival of larvae was determined after hatching by

calculating the percentage of larvae that were motile (alive) and those that were not (dead) out of the total larvae that hatched (Thoithi et al., 2002).

Identification of the eggs

The eggs of *Ancylostoma caninum* are typical strongyle with an ovoid shape and a thin wall. They measure about 40x65 µm, and contain already 4 to 16 cells when shed with the feces. The eggs of *Toxocara canis* are almost spherical, brown in color and measuring about 50x85 µm. They have a thick wall with a rough surface, and contain a single cell when in feces. (www.parasitipedia.net)

Preparation of the eggs solution

According to Coles et al. (2006) eggs intended for the hatching test should be used within 3 h of collection because sensitivity decreases with age of the eggs. To obtain fresh fecal samples, the puppies were given dog food pellets at 8 am on the day of harvesting, after starving them for 12 to 18 h; this ensures that they defecate within an hour of feeding releasing fresh samples.

About 200 g of a pooled fecal sample was mixed into a suspension with saturated saline solution (350 g of NaCl in 1,000 ml of tap water) and allowed to settle for 30 minutes. A plastic petri dish was placed on the surface of the suspension in order to allow the eggs adhere to the bottom of the dish. The eggs were recovered by washing off with distilled water then centrifuged twice in order to remove the excess salt. 15 ml of distilled water was added to the final residue and mixed well. 50 µl of this solution were dropped into three microscope slides and a cover slip placed onto each and then observed under a light microscope at X10 magnification in order to count the number of strongyle and ascarid eggs in each slide. The average estimated number of eggs per 50 µl of the parasite egg solution was 173 strongyle and 27 ascarid eggs.

Exposing the eggs to the extracts

The extracts were reconstituted by adding 40 mg of each dry extract into 2 ml of distilled water in order to make a stock solution. The aqueous extracts of each of the plant was dissolved readily in water while the ethanol extracts of *A. sativum* and *A. cepa* dissolved after raising the temperature of the mixture in a water bath at 40°C for 15 min and shaking vigorously. The ethanol extract of *J. curcas* could not dissolve in water even after shaking or raising the temperature. Therefore it was not used for any tests.

The stock of 20,000 µg/ml of each plant extract was diluted serially to give 10,000, 5,000, 2,500, 1,250, 625, 312.5 and 156.25 µg/ml in a microtitre plate in order to obtain 150 µl of each dilution per well. A total of 50 µl of the parasite solution was added into each well to make a final mixture of 200 µl of parasite eggs and extract in each well. Further dilutions of 78.13, 39.06, 19.53, 9.77, 4.88, 2.44, 1.22 and 0.61 µg/ml were done for ethanol extracts of both *A. cepa* and *A. sativum* and for the aqueous extract of *A. cepa*. For the positive control a similar dilution was made as for the drug preparation (Vermic Total™) containing a combination of febantel and pyrantel permeate which are both sensitive to *A. caninum* and *T. canis*. For the negative controls, one well had 200 µl of the parasite egg solution and another one had 150 µl of 99.5% ethanol mixed with 50 µl of the parasite egg solution. The microtitre plate cover was replaced, wrapped up with aluminum foil and incubated for 2 weeks. Observations were made under an inverted microscope after 48 h and again after 96 h for *A. caninum* eggs then on alternate days up to 2 weeks for *T. canis* eggs. This is because *A. caninum* eggs usually hatch after 48 to 72 h into free

Table 1. Anthelmintic activity of water and ethanol extracts of bulbs of *Allium cepa*, *Allium sativum* and leaves of *Jatropha curcas* against *Ancylostoma caninum*.

Concentration (ug/ml)	Plant extract eggs hatch inhibition (EHI) and larval motility assay (LM) against <i>A. caninum</i>									
	<i>A. cepa</i>				<i>A. sativum</i>				<i>J. curcas</i>	
	Aqueous (%)		Ethanol (%)		Aqueous (%)		Ethanol (%)		Aqueous (%)	
	EHI	LM	EHI	LM	EHI	LM	EHI	LM	EHI	LM
10,000	99	70	100	0	13	93	99	0	11	94
5,000	70	79	100	0	9	90	100	0	7	97
2,500	63	77	100	0	8	92	98	0	8	99
1,250	53	84	97	0	4	95	99	0	5	100
625	24	88	89	0	4	97	92	0	4	99
312.5	27	93	86	0	3	97	85	15	4	100
156.25	15	95	80	0	3	100	78	17	2	100

Table 2. Anthelmintic activity of water and ethanol extracts of bulbs of *A. cepa*, *A. sativum* and leaves of *J. curcas* against *T. canis*.

Concentration (ug/ml)	Plant extract eggs development inhibition (EDI) against <i>T. canis</i>				
	<i>A. cepa</i>		<i>A. sativum</i>		<i>J. curcas</i>
	Aqueous (%)	Ethanol (%)	Aqueous (%)	Ethanol (%)	Aqueous (%)
	EDI	EDI	EDI	EDI	EDI
10,000	78	100	8	90	5
5,000	54	100	7	89	4
2,500	50	100	7	88	5
1,250	43	100	6	82	3
625	4	90	6	79	3
312.5	3	88	5	75	2
156.25	0	80	3	70	2

larvae while *T. canis* eggs develop to a fully larvated egg over a period of 2 to 6 weeks and does not break into free larvae (Dena et al., 2012). The readings were done by counting the number of free *A. caninum* larvae and the number of developing *T. Canis* eggs. The percentage inhibition was calculated using the following formula;

$$\frac{x-y}{x} \times 100$$

Where: X is the estimated total number of eggs per 50 µl; Y is the number of hatched or developing eggs.

RESULTS

Table 1 shows the percentage inhibition of egg hatching and effects on motility of larvae of each of the six extracts on *A. caninum*. Table 2 shows the percentage Egg Hatch Inhibition on *T. canis* and Table 3 shows the dose response on further dilution of aqueous and ethanol extracts of *A. cepa* and ethanol extract of *A. sativum*. The aqueous and ethanol extracts of *A. cepa* inhibited hatching of eggs of both *A. caninum* and *T. canis* and has moderate effect on survival of larvae as shown in Tables

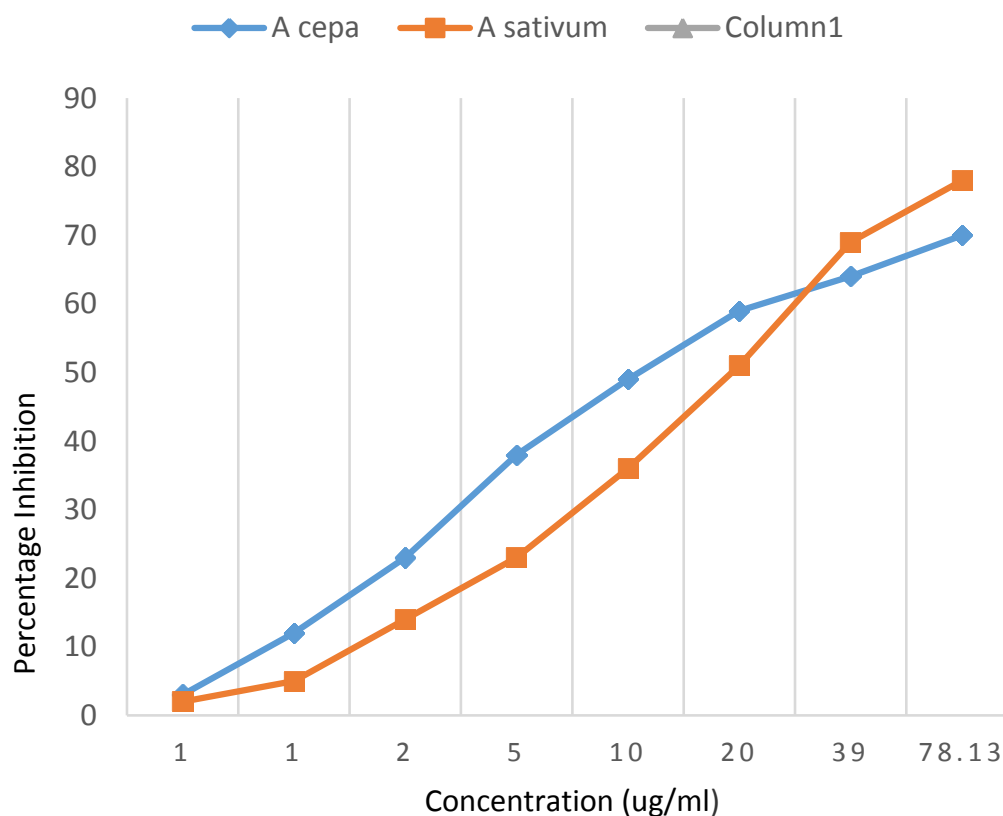
1 and 2. However, the ethanol extract gave higher percentage inhibition rates at lower concentrations than the aqueous extract. This suggests that there was a better dose response for the ethanol extract of *A. cepa*. Ethanol extract of *A. sativum* also inhibited hatching of eggs of both parasites and had moderate effect on survival of larvae. Both aqueous extracts of *A. sativum* and *J. curcas* did not have effect on the eggs and on survival of larvae as shown in Tables 1 and 2. Figures 1 and 2 compare the percentage inhibition of ethanol extracts of *A. cepa* and *A. sativum* on *A. caninum* and *T. canis* respectively. Both show that the ethanol extract of *A. cepa* has a higher dose response. None of the extracts was as active as the combination drug VERMIC TOTAL™ at equal concentrations.

DISCUSSION

This work reports for the first time the in vitro anthelmintic activity of the water and ethanol extract of bulbs of *A. cepa* and *A. sativum* as well as the leaves of *J. curcas* on *A. caninum* and *T. canis*. The results indicate that the

Table 3. Anthelmintic activity of ethanol extracts of bulbs of *A. cepa*, and *A. sativum* and against *A. caninum* and *T. canis* on further dilution.

Concentration (ug/ml)	Percentage egg hatch/development inhibition				Percentage larval motility	
	<i>A. caninum</i>		<i>T. canis</i>		<i>A. caninum</i>	
	<i>A. cepa</i>	<i>A. sativum</i>	<i>A. cepa</i>	<i>A. sativum</i>	<i>A. cepa</i>	<i>A. sativum</i>
78.13	70	78	72	68	0	28
39.06	64	69	65	59	2	32
19.53	59	51	57	43	5	38
9.77	49	36	42	28	12	46
4.88	38	23	31	15	25	56
2.44	23	14	18	10	40	76
1.22	12	5	7	9	51	80
0.61	3	2	4	8	55	83

**Figure 1.** Dose response curve showing the egg hatch inhibition of ethanol extracts of bulbs of *A. cepa* and *A. sativum* on *A. caninum*.

ethanol extracts of *A. cepa* and *A. sativum* have anthelmintic properties against *Ancylostoma caninum* and *Toxocara canis*. There was a complete inhibition of Egg Hatching and Egg Development up to a dilution of 1,250 ug/ml for the *A. cepa* ethanol extract after which a dose response was observed. This percentage inhibition was higher compared to that of the ethanol extract of *A. sativum* suggesting that the *A. cepa* extract has a higher

anthelmintic efficacy. Both extracts showed good anthelmintic activity since they affected the survival of larvae. However, none of these extracts was as active as Vermic Total™ (Febantel and Pyrantel Permoate). The aqueous extract of *J. curcas* had no effect in terms of inhibition of egg hatching or development but had high activity against survival of the larvae. The sequential activity on both eggs and larvae as shown by the ethanol

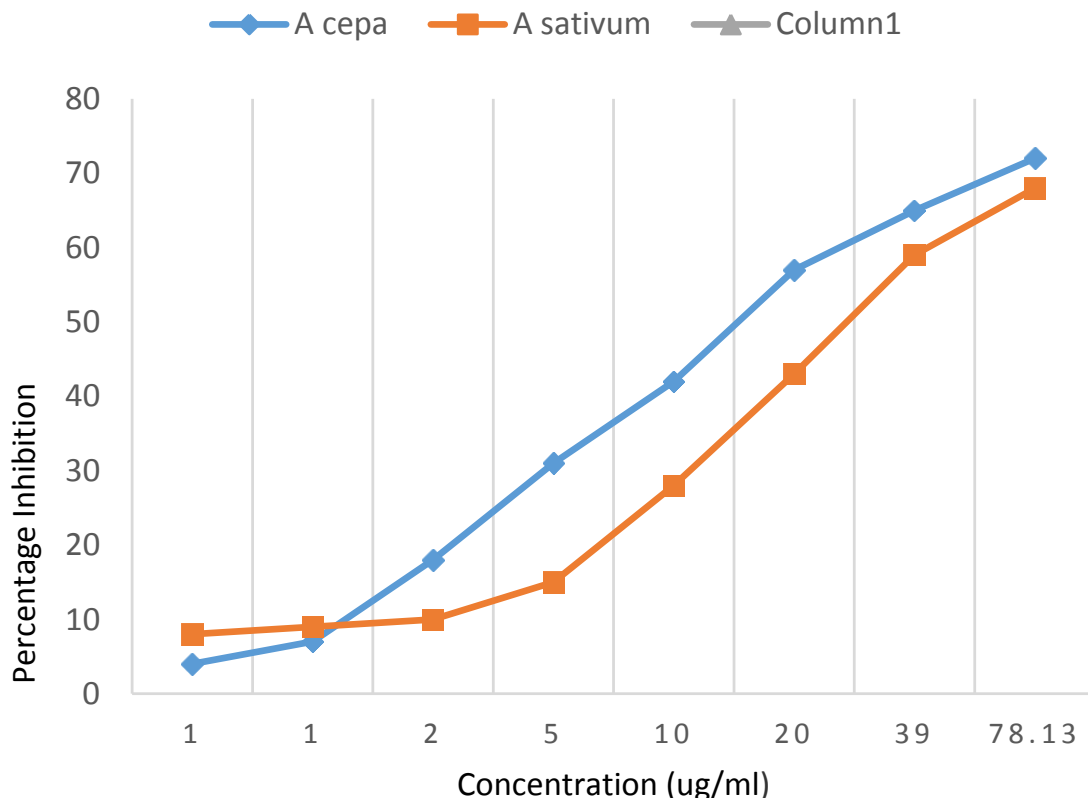


Figure 2. Dose response curve showing the egg development inhibitory effects of ethanol extracts of bulbs of *A. cepa* and *A. sativum* on development of *T. canis* eggs

extracts of *A. cepa* and *A. sativum* as well as the aqueous extract of *A. cepa*, is comparable to that of commonly used anthelmintic drugs like benzimidazoles which are active against eggs, larvae and adult stages of nematode worms (Behm and Bryant, 1983). The results support the work of other researchers who reported that crude extracts of *A. cepa* and *A. sativum* have anthelmintic properties (Abhijeet et al., 2012; Mantawy et al., 2012; Kumar, 2014; Zafar, 2014). Dimethyl Sulfoxide (DMSO) or Tween80 can be used to concentrate organic solvent dry extracts which cannot be reconstituted in water. However according to Luciana et al. (2011) worms can tolerate up to 2% DMSO or Tween 80 although even these solvents at 2% cannot dissolve extracts made with pure ethanol. Therefore this study did not use DMSO or Tween 80 to reconstitute the ethanol extract of *J. curcas* but opted to remove it from the experiment.

The use of 20,000 ug/ml as the concentration of stock solutions for serial dilution of each extract was based on methods described by Thoithi et al. (2002). Higher concentrations of aqueous extracts of *A. sativum* and *J. curcas* may show better effect on egg hatching and survival of larvae. More work can be done to test the in vitro effects of these extracts at higher concentration ranges. Further pharmacological work should be carried out on the efficacy of the ethanol extracts of *A. cepa* and

A. sativum in puppies to determine the toxic effects in mice in order to get further information that can support the use of the medicines for control and treatment of worm infestations in dogs.

Conflict of Interests

The authors have not declared any conflict of interest.

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