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Toxicity of *Toddalia asiatica* is associated with microcephaly and hypochondroplasia in mice

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ABSTRACT

In Kenya various plant extracts are widely used as folklore remedies for various ailments including malaria etc. However, use of these traditional remedies poses a risk to the users due to the scarcity of data on their safety including their teratogenic potential. Hence this study evaluated the teratogenic and sub-acute toxic effects of methanol: dichloromethane extract of *Toddalia asiatica* in animal mice. In the study, young female mice aged between 6-7weeks were mated with mature males and conception confirmed using vaginal plug, maternal weight and breast enlargement. Administration of the dosed of the plant extract and controls was done from day 6 through 15 of gestation. The mice were euthanized on the 19th day of gestation. The weight of the gravid uterus, pups, number of pups and gross examination to identify any physical abnormalities was done. In the toxicity study male mice aged between 6-7 weeks received oral administration of high doses of plant extract and the control daily for 28 days. On the 29th day animals were sacrificed, cardiac puncture conducted and collected blood for hematology and biochemistry analysis. The data obtained was analyzed using one way ANOVA followed by *Tukey* as the post hoc test. Statistical significance was set $p < 0.05$. The extracts caused significant reduction ($p<0.001$) in head size, birth weights and length of the limbs but no effects on hematology parameters. Hence the plant extract exhibited teratogenic effects and thus should be used with care during pregnancy.

Keywords: Teratogen, *Toddalia acacia*, Microcephaly, Hypochondroplasia.

INTRODUCTION

Birth defects are universally recognized health problem which exist at birth. The 63rd world health assembly requested member states to continually detect and prevent causes of birth defects. Prevalence of congenital malformations varies from region to region due to the diverse nature of the causative agents. In Egypt it is recorded at 2.5% [1], Kenya1.94% [2]. Globally 70% of birth defects are idiopathic [3]. Emerging and re-emerging infectious diseases and ability of most infectious micro-organism to resist modern medicine are in the rise which further exacerbate the burden of diseases in the communities [4]. Herbal medicine have been in use since ancient days and they are increasingly being used due to their affordability, ease of access and the narrative that they can treat many diseases with few side effects [5, 6]. It is estimated over 80% of the world population uses herbal medicine [7], this has led to exposure of more substances whose safety and teratogenic potentials are unknown. *Toddalia asiatica* (Rutaceae) Orange climber in English, *Mururue or mwikunya* in Kikuyu, and *Oleparmunyo* in Masai [7] is a liana with root that have a bitter taste [8] and thrives well in many tropical and subtropical countries of Africa and Asia [9]. It is widely used in East Africa as folklore remedy for management of pain and inflammation [10, 11], and malaria [12, 13], a leading global health problem [7]. Other pharmacological uses include; anti-inflammatory, analgesic effects, bacteriostatic, antioxidant and an anticancer [14]. However, there is paucity of data on its toxic and teratogenic effects. The plant contains coumarins, alkaloids, terpenoids and flavonoids [14]. Despite the increased use of plant as folklore remedies, information on the safety during pregnancy is lacking. Hence this study aimed at evaluating its teratogenic and toxicity effects using animal model.

MATERIALS AND METHODS

Plant collection

The plants materials were collected from Subukia area of Nakuru County, Kenya about 200kms North West of Nairobi on July 17, 2021 around 10:30am. They were packaged in cartons and transported to Kenyatta University Biochemistry, Microbiology and Biotechnology Department animal house for processing and analysis. A sample of the specimen was taken to University of Nairobi herbarium for botanical authentication and voucher number JG2021/01TA was issued.

Preparation of the extract

Toddalia asiatica leaves were air dried at room temperature away from direct sunlight for two weeks. They were then grounded to powder using an electric mill (Zhengzhou Yize machinery co., Ltd. Henan, China). Then 100 grams of the powdered plant was soaked in 400mls of methanol, dichloromethane mixture in the ratio of 1:1and the extraction process repeated three times by replacing saturated solvent after 30mins and 2hrs then allowed to stand overnight before decanting and filtering. The filtrate was evaporated at 62ºC using a vacuum evaporator to obtain a concentrated plant extract of about 3grams. The final product was transferred into McCartney bottles and refrigerated for use in downstream experimental processes.

Experimental animals

Virgin female mice aged between 6-7 weeks and weighing about 20-25gm and males aged between 12-14 weeks were used. Before animals were allowed to mate they were housed in cages for 7 days at room temperature for acclimatization. Water and rodent pellets were provided *at libitum.* All experiments were done in compliance with guidelines for care and use of laboratory animals [15].

Drugs and chemicals

Drugs and chemicals used in the study included normal saline, methanol, dichloromethane, dimethyl sulfoxide, chloroform and phenytoin sodium.

Bioassays

In the study, young female mice aged between 6-7weeks were mated with mature males and conception confirmed using vaginal plug, maternal weight and breast enlargement. Administration of the dosed of the plant extract and controls was done from day 6 through 15 of gestation. The animals were randomly assigned into four groups (n-5). Group one was orally administered 0.2mls of the vehicle (10% DMSO in normal saline) daily from the 6th through the 15th day of gestation. On the 19th day the mice were weighed then euthanized using chloroform and pups removed by ceasarean section. The same procedures were repeated using 100 and 500mg/kg doses of *Toddalia asiatica* and phenytoin sodium. The following parameters were recorded; maternal weight prior to sacrifice, weight of uterus and pups, number of live pups, weight of pups, length of pups, head circumference, length of the fore legs, hind legs and fetal resorption.

Toxicity study involved use of male mice aged between 6-7 weeks which involved orals administration of 0.2mls of the 100 and 500mg doses of extracts and the vehicle daily for 28days. On the 29th day animals were sacrificed, cardiac puncture blood collection was done and used in hematology and biochemistry testing.

Phytochemical screening

Phytochemical screening was carried out using methods described by Shaikh & Patil 2020. The results of the reactants were noted by change of color of the reactants [16].

Data analysis

The data was analyzed using one way analysis of variance (ANOVA) followed by Tukey as the post hoc test. Statistical significance was set at $p < 0.05$ and 95% confidence interval.

RESULTS

Table 1: Effects of *Toddalia asiatica*extracts of on mice pups

	Pup length (mm)	Head circumference (mm)	Head size (mm)	Foreleg (mm)	Hind leg (mm)
Vehicle	42.58±0.55	7.21±0.04	11.15±0.033	7.65±0.019	10.19±0.019
100mgs	38.53±0.101 *	6.81±0.051 **	10.89±0.039	7.489±0.05	9.97±0.029
500mgs	40.39±0.53 *	6.74±0.068 **	10.76±0.091	7.29±0.058	9.83±0.084
p value	0.001	<0.001	0.012	0.003	0.009

Values in the tables represent length in millimeters. *p < 0.05, **p < 0.001 against the vehicle.

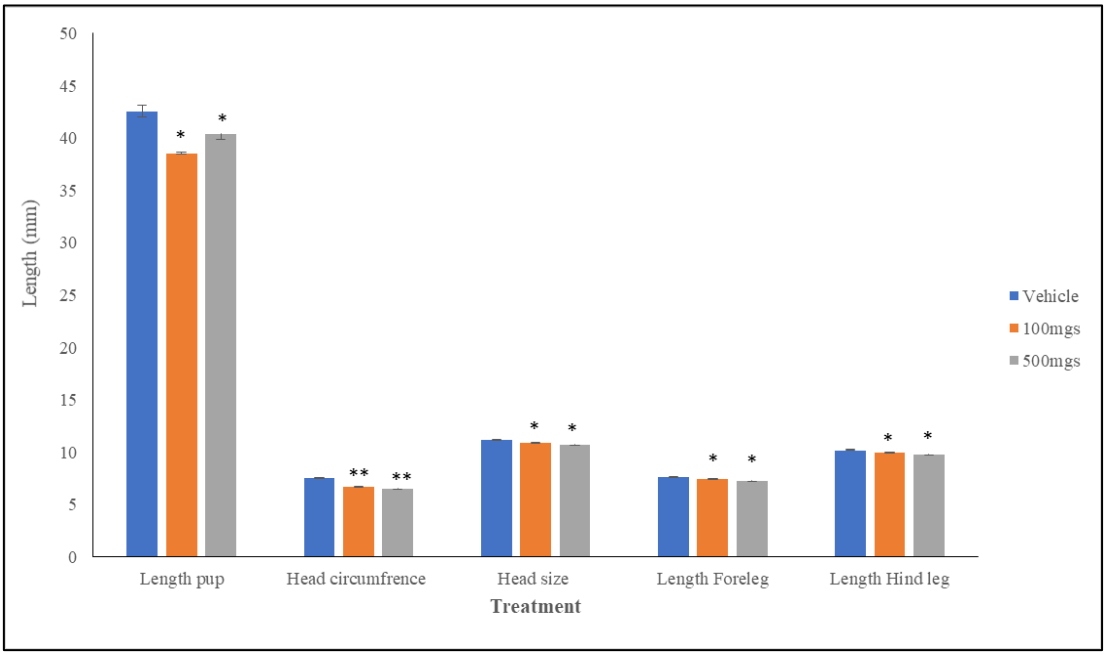

Figure 1: The effects of extracts of *Toddalia asiatica* extract on body part lengths on mice pups. *p < 0.05, **p < 0.001

Table 2: Effects of *Toddalia asiatica* extracts on liver enzymes in mice

	ALT	AST	GTT	ALP
Vehicle	54.72±9.2	400.33± 31.76	267.5± 10.84	0
100mgs	53.07±5.09	291.37±11.88**	0.61± 0.38*	6.21 ± 3.76
500mgs	69.33±5.87	517.23±14.21**	2.83±1.5*	0
p value	0.224	<0.001	0.001	0.0966

Values in the table show enzyme levels bilirubin and their standard errors. *p< 0.05, **p< 0.001 versus the vehicle.

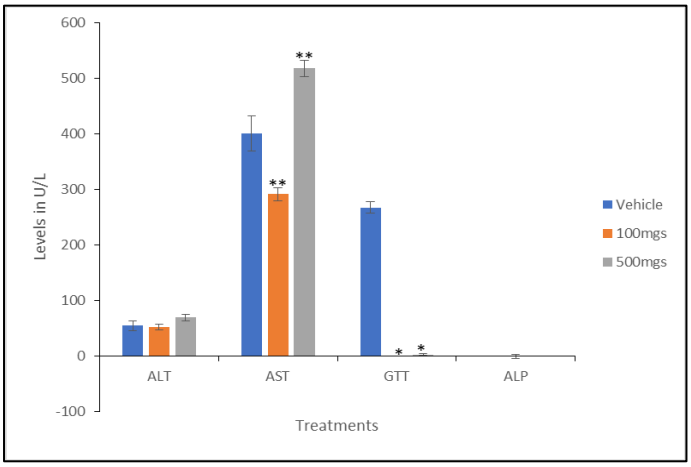

Figure 2: Effects of *Toddalia asiatica* extracts on hepatic enzymes levels in mice. *p < 0.05, ** p < 0.001 versus the vehicle

Table 3: Effects of *T. asiatica* extracts on mice bilirubin levels

	Total Bilirubin	Direct Bilirubin	Indirect Bilirubin
Vehicle	5.62±1.22	4.29±1.15	1.47±0.29
100mgs	5.07±0.88	3.38±0.42	1.69±0.58
500mgs	10.97±0.66**	7.93±0.52*	3.04±0.49
p value	<0.001	0.0018	0.0656

Values in the table show levels bilirubin in micromoles per liter and their standard errors. *p< 0.05, **p< 0.001 versus the vehicle.

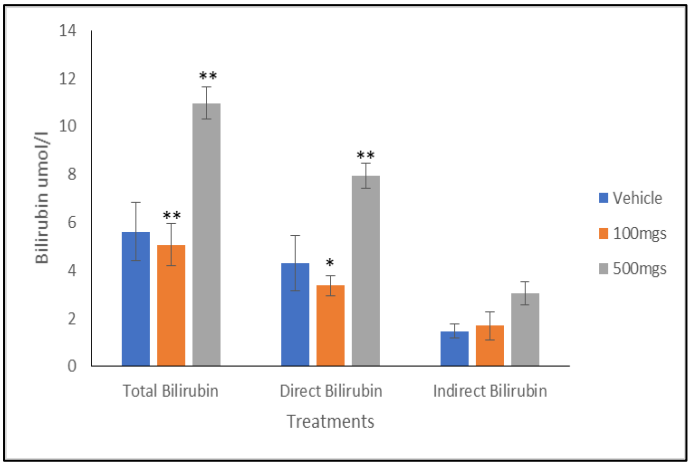

Figure 3: The effects of *Toddalia asiatica* extracts on bilirubin levels in mice. *p < 0.05, **p < 0.001

Table 4: Effects of *Toddalia asiatica* extracts on levels plasma proteins in mice

	Total Protein	Albumin	Globulin
Vehicle	91.5±15.6	32.27±4.21	59.23±13.24
100mgs	68.45±4.45*	25±0.60*	43.45±4.13*
500mgs	151.95±5.18**	53.77±4.0**	98.18±3.66**
p value	<0.001	<0.001	<0.001

Values in the table represent weights of plasma proteins in grams. *p < 0.05, **p < 0.001versus the vehicle

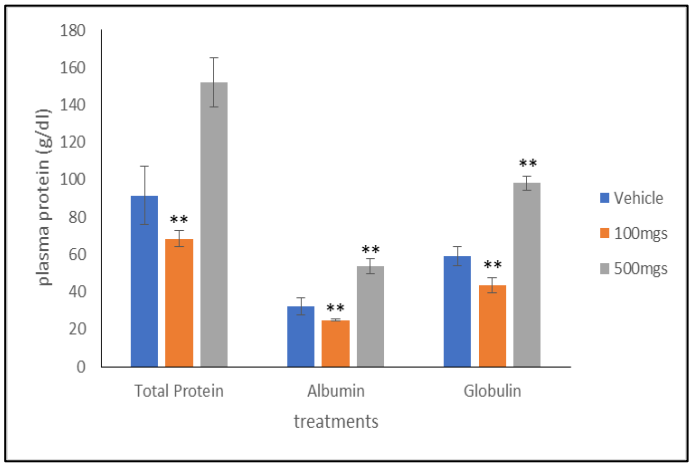

Figure 4: Effects of *Toddalia asiatica* extracts on levels plasma proteins in mice. ** (p < 0.001)

Table 5: Show the qualitative phytochemical profile of*Toddalia asiatica* extracts

Phytochemical	Status
Alkaloids	+
Cardiac glycosides	+
Flavonoids	+
Tannins	+
Saponin	+
Terpinoids	+
Coumarin	+

Status: +Present - Absent

DISCUSSION

Toddalia asiatica is extensively used as folklore medicine for various ailments such as malaria body pains [9] with little or no information on its teratogenic potential. However, the results showed that mice treated with the extracts from the plant caused significant reduction in head sizes or microcephaly. Microcephaly is characterized by small headsize compared to normal headsize from pups of the same age. There are many causes, some known and others unknown [17]. Known causes includes exogenic factors like intrauterine infections and exposure to known teratogens such heavy metals like arsenic, mercury, alcohol, and smoking [11]. Genetic causes of microcephaly include numerical chromosomal aberrations or micro-deletions or duplication problems like trisomy 13 (Patau syndrome), 18 (Edwards' syndrome) and 21 (Down syndrome). Microcephaly may also occur due to single gene variations such as Seckel syndrome, X-chromosomal microcephaly, Rubinstein-taybi syndrome among others [17]. It also prudent to say that may be some of the idiopathic causes could be due to consumption of some of these untested plant remedies.

Both the fore limb and hind limb length were also significantly reduced ($p<0.05$) following the treatments (Fig 1; Table 1). This condition is known as hypochondroplasia [18]. Herbal remedies have been shown to interfere with limb development. For example, Wabai et al [19]. Other cause of abnormal limb development document includes the legendary thalindomide [20] the subacute toxicity of *T. asiatica* extract involved measurement of the concentration of hepatic enzymes, bilirubin, plasma proteins, creatinine, and urea. The results showed an increase in the levels aspartate amino transferase (AST) and a decrease in gamma glutamyl transferase (GGT). There was no effect on the levels of Alanine aminotransferase (ALT) and alkaline phosphatases. Substance like lipids, vitamins, hormones, minerals and drugs are transported in the body bound to plasma proteins through substance specific receptors. Serum albumin is generally higher, almost two folds as compared to serum globulin however, in this study globulin levels were higher than albumin (Fig 4; Table 4). This may occur during infections, autoimmune diseases and cancer [18]. Aspartate aminotransferase (AST) is found in the liver, muscles, heart, kidneys and red blood cells. GGT is elevated in alcohol abuse and biliary tract disease. ALT is primarily found in the liver thus a specific marker in assessing liver functionality. Since ALT was not affected, it indicates that the plant extract does not induce damage to the liver cells however, there might be some effects on other body tissues and organs such as the muscles, heart, kidneys or red blood cells which culminated to high levels of AST detected. Levels of alkaline phosphatase were not affected. (Fig 2; Table 2).

There was an association between administration of the plant extract ($p<0.05$) and an increase in levels of conjugated bilirubin but not on the levels of unconjugated bilirubin (Fig 3; Table 3). Presence of elevated levels of conjugated bilirubin always indicate there is a problem in the body which need to be evaluated further to get to the root cause. It is an indication the liver if conjugating bilirubin but it's distribution and extraction is being interfered outside the liver. The condition is commonly seen in biliary system disease. Results produced also gave low levels of GGT 0.16U/L and 2.83U/L for *T. asiatica* 100 and 500mgs respectively (Fig 2; Table 2), which ruled out chances of having biliary tract disease. High concentration of plant extract administration led to an increase in the levels of plasma proteins (Fig 4; Table 4) but the ratio remained unaltered.

Table 5 shows the phytochemical present in the plant. Cold extraction was done, there is need to use hot extraction processes and evaluate whether there are more phytochemicals which could be present in the plant. Further studies are needed to evaluate the mechanisms of this phytochemicals which could lead to the teratogenic effects seen.

CONCLUSION

Extracts of *Toddalia asiatica* have teratogenic effects as indicated by the presence of microcephaly and hypochondroplasia in both treatment groups. Consumption of *Toddalia asiatica* plant extracts during pregnancy should be taken with caution.

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Conflict of Interest

None declared.

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