ISSN 2229-5518

Antimicrobial Activity of Indigenous Plants Used by Pastoral Communities for Milk Preservation in Kilosa District, Tanzania

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Abstract-- Milk is an important part of the diet among pastoral communities in Kilosa district, Tanzania. Its production and consumption derive much food security and economic benefits to the rural people in the district. However, Milk has a complex biochemical composition, and its high-water activity and nutritional value serve as an excellent medium for the growth and multiplication of many kinds of microorganisms. In order to improve the shelf-life and safety of milk, this study was explored. A cross-sectional study by use of 50 questionnaires was conducted on the knowledge and practices of the herbs used in milk preservation in two villages, after which plant parts of *Dalbergia melanoxylon* and *Combretum imberbe* were collected for laboratory analysis. Biological activity using Minimum inhibition concentration (MIC) serial dilution assay against six milk spoilage bacteria and Titratable acidity of raw milk treated with plant extracts were determined. Descriptive statistics and one-way analysis of variance was used to analyze the data with the aid of Microsoft Excel package 2016. The common methods for preservation of milk were boiling (26%), and, fumigation of milk containers using herbs (74%). Ethanol extracts of the various plant parts showed broad-spectrum antimicrobial activity against *Escherichia coli, Salmonella typhi, Pseudomonas aeriginosa, Staphylococcus aureus, Streptococcus agalactiae* and, *Proteus spp*. However, there was no statistical difference in lactic acid production between milk samples in vivo. The results of this study gives credence to the traditional use of *Dalbergia melanoxylon* and *Combretum imberbe* and expand knowledge on the biological activity of their extracts as potential natural preservatives.

Index terms: *Combretum imberbe*, *Dalbergia melanoxylon*, Food safety, Indigenous knowledge, Medicinal plants, Milk, Tanzania

1.0 Introduction

Milk contains a balanced form of all the necessary and digestible elements for building and maintaining the mammalian body [1]. It has however a complex biochemical composition and its high-water activity and nutritional value serve as an excellent medium for the growth and multiplication of many kinds of microorganisms when suitable conditions exist [2]. Because of the hand milking system practiced among the pastoral communities and the low hygienic status of the udder from kraal debris at the time of milking, it is not easy to avoid contamination of milk with microorganisms [3, 4].

The safety of raw milk is of worldwide concern where various efforts are directed since it is associated with food-borne diseases [5]. Spoilage of milk occurs as a change of flavor, undesirable coagulation of milk proteins, and the increased concentration of free fatty and amino acids [6]. The determinants of the shelf life of fresh milk are usually the spoilage bacteria that have the ability to grow at different temperatures. Microbial growth induces changes in the taste and odor of milk such as sour, putrid, bitter, malty, fruity, rancid and, unclean [7]. Several microbes have been reported to cause milk and dairy products spoilage. Among them include *Pseudomonas* spp and *Bacillus* spp which are the most commonly isolated organisms in raw or heat-treated (pasteurized) milk at the time of spoilage. Other bacteria such as *Bacillus cereus*, *Salmonella* spp. *Staphylococcus aureus* and, *Escherichia coli* are known to be common contaminants of milk and dairy products that produce different enterotoxins responsible for human infections or intoxication [8]. Coliforms can cause rapid spoilage in milk because they ferment lactose with the production of acid and gas, and they can also degrade milk proteins. *Escherichia coli* is a well-known example of coliform [9].

Though several techniques are known to be used in the process of milk preservation such as heating and cooling to delay milk spoilage, the major problem facing pastoralists is the absence of infrastructure to maintain a cold chain, ignorance of modern food preservation techniques and financial constraints which hinder the resource-poor actors to access appropriate technology [11]. The use of traditional plants is of tremendous importance in many societies, including most rural African communities. In several pastoralist communities, the uses of medicinal plants are the most commonly used technique for different food preservation including milk [12]. According to Burt [13] several plants, herbs and spice extracts have been reported to possess antimicrobial activity against a range of bacteria, yeast, and molds.

Concerns about milk spoilage, post-harvest milk loss and human health risks, need to be addressed in the context of consumer practices, such as the use of indigenous plants, to delay or eliminate potential microbes. Furthermore, despite the important contribution of cow milk to pastoralists, little is known about the postharvest handling, preservation and processing methods of Pastoralist in Kilosa district. This study, is, therefore, aimed at exploring existing indigenous knowledge, skills and, practices associated with the use of herbs for milk preservation among Pastoralist households in Kilosa District. Indigenous knowledge is the basis for local-level decision-making about vital technologies that are acceptable to their lifeways. It evolves from the experiences of farmer's found to possess practical utility in solving some of the farmer problems under their own conditions. Indigenous knowledge is often an untapped resource that can be applied and scientifically validated [14] [15]. This can result in developmental solutions that are acceptable in local communities. In this study understanding, local practices will help in designing appropriate strategies to enhance the contribution of cow milk to food security for the pastoral communities, especially the poorer households. The study assesses indigenous knowledge and antimicrobial activity of traditional herbs used in the preservation of milk in Kilosa District, Tanzania.

2.0 Materials and Methods

2.1 Study Area

The study was conducted in Kilosa District, Morogoro Region, and east of mainland Tanzania. The district lies between latitude 5 °55´ and 7°53´ to the South of the Equator and longitude 36°30´ and 37°30´ east of Greenwich Meridian. [16]. Kilosa district's Mbwade and Parakuyo villages were selected purposely to be used as study sites because milk is one of the predominant contributors to household income in the area. The area is dominated by the pastoralists of the Maasai tribe. These are Maasai of the nilotes tribe (people) known in East Africa and to the whole world as cattle keepers [17]. The map of the Morogoro region is shown in figure 1.

Figure 1: Map of Kilosa district Obtained from Google map (sited on 14th July, 2016 from http://www.maplandia.com/Tanzania/Morogoro/kilos)

Sampling designs, data collection and analysis

***2.2* Indigenous Knowledge and Ethnobotany Survey**

A structured questionnaire with open and closed-ended questions was used during the survey to gather information about traditional knowledge, practices, and plants used for milk preservation. The sample size was determined based on [18] formula $n= Z^2p\ (1-p) / d^2$ for sample size determination for an unknown population at a confidence level of 95%. A total of 50 women were selected for the study because milk handling is a preserve of women, it characterizes them in pastoral communities [19]. Maasai men are responsible for protecting and herding the cattle, women are in charge of milking the cattle and post milking treatment of the milk. Thus men were not included in the study. The sampling method for the inclusion of women in the study was purposive sampling. The inclusion criterion at the household level was the practice of pastoralism. The data was collected through personal interviews and direct observation of milk handling practices as practiced by the women. The questionnaire was administered by a researcher with the help of a Kiswahili interpreter, the district veterinary officer. The data collected was compiled and analyzed and two plant species collected from the community.

2.3 Determination of antimicrobial activity

Plant sample identification and collection

Leaves, stems, and roots of two plant species, *Dalbergia melanoxylon,* and *Combretum imberbe* were collected from Parakuyo village which had plenty of these tree species growing within the village. The two plant species were selected based on the results of the ethnobotanical cross-sectional survey. Before the collection of plant materials for further analysis, the plants were identified and authenticated by a botanist from the Department of Forest Biology, Sokoine University of Agriculture. The plant parts were cut out from the trees and dug out with the help of the interviewed households. The plant materials were air dried separately at room temperature for 7 days. Then the stems and roots were sliced into smaller pieces. The dry plant materials were ground to powder using a laboratory grinder to approximately 50 microns. This was done at the Institute of traditional medicine laboratory, Muhimbili University of Health and allied sciences in Dar es Salaam.

Plant Extraction

The extracts were prepared by cold maceration. 200g of each dried ground plant material was added to 1000 mL conical flasks, and 900 mL 96% ethanol solvent was added to each flask. Each mixture was left to extract for 48 hours at room temperature. Ethanol was used as the solvent of choice because it is a molecule with both a polar and nonpolar end therefore it can extract both polar and nonpolar secondary metabolites [20]. The plant extracts were filtered through Whitman No.1 filter paper. The filtrates were dried under vacuum using a flash rotary evaporator to obtain final masses of 1.94, 1.68 and 5.54g from the first plant species and 1.31, 1.78 and 7.56g from the second species [21]

Figure 2: Shows dissolved plant extracts in 1ml of DMSO

Microbial culture

A total of 6 milk spoilage bacteria strains were used in the study: *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 29953, Pseudomonas ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Streptococcus agalactiae* ATCC 27591. Conventional methods in the Mueller-Hinton Agar medium were used to prepare the cultures in a medical laboratory [22].

In-vitro Antimicrobial Assay

The micro plate serial dilution method [23] was used to determine the minimum inhibitory concentration (MIC) of extracts against six bacteria. Extracts of 10 mg/ml and 1 mg/ml were dissolved in DMSO and serially diluted with sterile water in micro plates in a laminar flow cabinet [24]. The same volume of an actively growing culture of the test bacteria in (0.5 Mcfarland standard turbidity) was added to the different wells and cultures were grown overnight at 37 °C [25]. The next morning 0.02% p-iodonitrotetrazolium (INT) chloride dye was

ISSN 2229-5518

added to all the wells. Growth was indicated by a pink color of the culture. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC while the negative control DMSO and Gentamicin as the positive control. Descriptive statistics and one-way analysis of variance was used to analyze the data with the aid of Microsoft Excel package 2016.

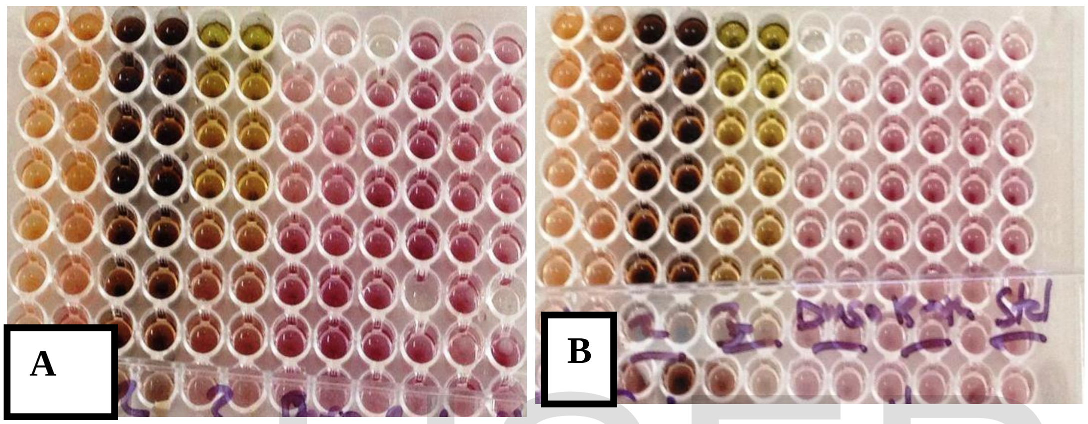

Figure 3: A 96-well plate showing minimum inhibition concentration (MIC) of different concentrations tested for *Salmonella* spp (A) and *Pseudomonas* spp (B)

2.4 In vivo biological activity of plant extracts in raw milk

The percentage titratable acidity was determined according to AOAC method 947.05. Plant extracts and milk preparations were separately titrated with standard alkaline reagent. All determinations were carried out in triplicate. Results were expressed as lactic acid equivalent using titratable acidity [26][27]. The data was analyzed using One-way analysis of Variance (ANOVA) by aid of Microsoft Excel package 2016.

ISSN 2229-5518

Figure 4: Milk samples treated with different plant extracts obtained from the test plants

3.0 Results

3.1 Ethnobotanical survey

3.1.1 Social economic status of the producers

The study included 50 women respondents who owned dairy cows and were willing to participate in the study. Majority of the respondents (72 %) were between the ages of 21-50 years old. About 14% of the respondents did not know their ages and 14% were above 50 years old. Most respondents (54%) owned less than 20 cows and the main cattle breed in the surveyed areas was the Short-horn Tanzanian Zebu. The pastoral women milk twice a day and the majority obtain 5 liters of milk per day or less. The majority (58%) used the milk for home consumption. For the 46% of respondents that were selling part of their milk, only 19% were able to sell their milk within the neighborhood. The majority (87%) were taking their milk to a long-distance market. The results show that there are no milk collection centers in either of the villages under investigation.

3.1.2 Local practices in post-harvest milk handling

This study shows that four practices were used by the pastoralist women to handle milk after milking. The milk was consumed raw by 16% of respondents, 84% of the women engaged in various milk handling practices. From the results of this study, it shows that only a few are still practicing the consumption of raw milk. The aggregate majorities are practicing other methods such as fermentation of the milk, others boil the milk before consumption, and others treat the milk by fumigation with selected plant species. The practices included boiling, Fermentation into sour milk, a local delicacy called (Mtindi), while the majority practiced milk treatment by fumigation of milk containers using selected plant species. The main post-harvest milk handling practices are illustrated in figure 3. The study findings indicated that the main practice (62%) is herbal milk treatment. The main species of plants that were established to be used in the process were *Dalbergia melanoxylon* and *Combretum imberbe.* The women use all the mentioned species depending on which species is available in storage or one that is easily accessible in the nearby bush. They also mentioned that the plant species differ in their characteristics and thus plant preference varies from one woman to the next. Two main reasons were mentioned for the milk treatment, these were that the plants added flavor to the milk and extended the shelf-life of milk (86%) and the use of herbs helped to reduce the effects of lactose intolerance in lactose intolerant individuals (14%). The women collect stems and or roots of the two plant species from the nearby bushes within the villages. If the plants parts are still fresh, they leave them to dry. Once they are dry they chop them into smaller pieces that can easily fit into the milk container usually made gourds. A gourd is a plant of the family Cucurbitaceae particularly *Cucurbita* and *Lagenaria* or the fruit of the two genera of Bignoniaceae"calabash tree". The milk containers are fumigated. This indigenous milk preservation technology is locally referred to as Msisilo. The gourds are fumigated with smoke from burned wood of specific tree species mentioned above. The fumigation is done by pushing the smoking chips of the burning stem inside the milk gourd and rubbing it on the inside of the milk container until the smoke dies out, this takes about 5 to 10 minutes. The residual charcoal pieces are brushed out with a brush. The gourd is inverted and tapped on the outside, the procedure is repeated one or two times. The process is illustrated in figure 3 above. Fresh cow milk to be stored is then poured into the gourd. According to the respondents, if properly fumigated, cow milk could remain fresh for 24 hours at ambient temperatures.

3.2 In-vitro Antimicrobial activity of *Dalbergia melanoxylon* and *Combretum imberbe* extracts

The highest *in-vitro* activity was exhibited by the stems of *Dalbergia melanoxylon* with an MIC of 1.24 mg/ml against *salmonella typhi* with the least activity been shown by the stems of *Combretum imberbe* with an average MIC activity of 4.17 mg/ml across bacteria. The minimum inhibitory concentrations of the different plant extracts is shown in figure 7 below. There was no statistical difference between the antimicrobial activity of the plant extracts against all bacteria and all the bacteria showed no significant difference in their susceptibility to the extracts P=0.05

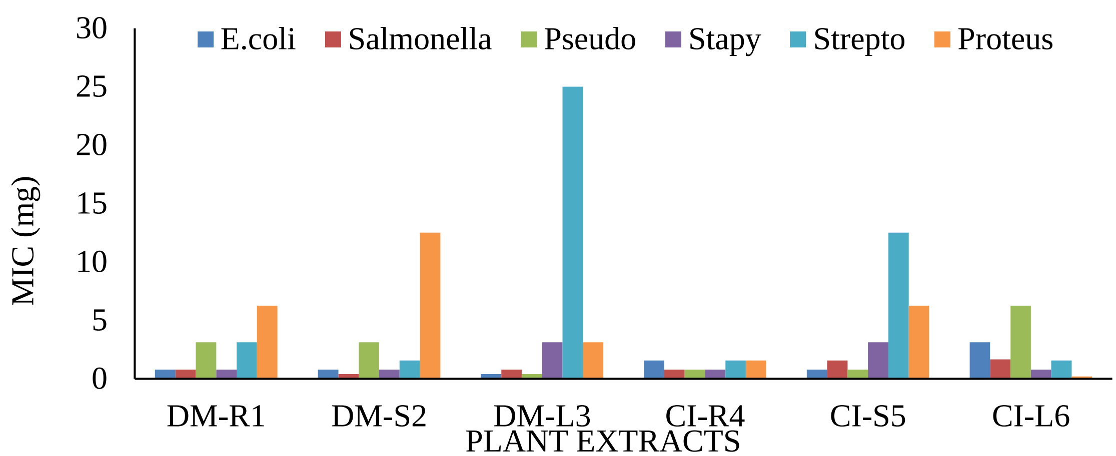

Figure 5: Antimicrobial Activity of the different plant extracts tested against different bacteria species using ethanol extracts

3.3 Titratable acidity in fresh milk samples

This study showed continuous increase in titratable acidity in all the samples subjected to 0.5, 1.0 or 10 mg/ml of the plant extracts. After 22 hours, the acidity ranged from 0.29%-0.55% regardless of whether the milk was treated with a specific plant extract or not. The lowest titratable acidity was observed in the leaves of *Dalbergia melanoxylon* leaves although there was no statistical difference between plant extracts, P=0.05.

4.0 Discussion

Although the women that participated in the study were of varying ages with the highest age range being less than 30 years of age, it was also observed that all the women shared similar knowledge with regards to post-harvest milk handling. This shows evidence of passage of knowledge from one generation to the other. The main preservation methods for fresh milk included fumigation of milk containers, fermentation and boiling of raw milk. Though the women mentioned various plants, there were only two plant species commonly used by the women in these villages. The plant species were identified to be *Dalbergia melanoxylon* and *Combretum imberbe* whose Kiswahili names are Mpingo [28] and Mtagalala respectively. The plant parts that were found to be of importance for the milk treatment were mainly the stems of the mentioned plants. The dried stem cuttings of these plants were used in the fumigation of the insides of the milk gourds used for milk storage with a purpose of enhancing the aroma and longer shelf life of the milk. Furthermore, fumigated milk was tolerable to milk intolerant individuals. Combined use of the plants was not reported instead one plant species is used at a particular time. The most preferred was *Dalbergia melanoxylon* for both flavor and preservation of milk freshness. The smoke sterilizes the gourd indirectly preserving the milk. The smoke lines the inside of the gourd, reducing its porosity rendering it airtight. This practice is known among the pastoral communities in different parts of Eastern Africa [29]. The procedure of milk treatment (*msisilo*) is similar to those reported in studies done in Kenya. According to Mureithi [30] thus milk treatment technology originates from the Kalenjin Community of Kenya for whom milk is a staple diet. The technology evolved as a result of the need to avoid wastage by preserving and storing excess milk for use during the dry season.

Results in this study revealed that the extracts which were extracted using a polar solvent, ethanol, were active against both gram positive and gram negative bacteria tested which are a common cause of spoilage and contamination in milk. This showed that the extracts of these plants are broad spectrum in their activities, thus they can act on against a wide range of milk-spoilage bacteria both gram-positive and gram-negative. From the results obtained, the most susceptible bacteria to the extracts were *Salmonella typhi* followed by *Escherichia coli*, both of which are gram-negative. The least susceptible was *Streptococcus agalactiae*. Gram-negative bacteria being more susceptible to the plant extract than Gram positive bacteria contradicts the previous reports that plant extracts are more active against Gram positive bacteria than Gram negative bacteria [31]. The observed antimicrobial activity of the plant extracts is an indication of presence of secondary metabolites. Secondary metabolites are small organic molecules manufactured by plants that make them competitive in their own environment. These small molecules exert a wide range of biological effects on the plant itself and on other living organisms. The biological effects provide the scientific basis for the use of herbs as preservatives, insecticides and, in traditional medicine in many communities. The organic compounds in *Combretum imberbe* were reported by Angeh [32]. Likewise, the occurrence of isoflavones, isoflavanones, neoflavones, sterols, anthraquinones, cinnamyl esters and triterpenes in *Dalbergia melanoxylon* has been reported by Mutai [33]. However, the combined effect of these extracts was not studied during this study. This result not only show the scientific basis for the traditional use of these plants in traditional milk treatment, but also confirms that ethnobotanical approach is a valuable approach when investigating antimicrobial properties of plants.

Based on the *in-vitro* antimicrobial activity of the plant extracts *D. melanoxylon* and *C. imberbe*, it was logical to consider in vivo application of the plant extracts in the milk as preservatives because results obtained *in vitro* cannot usually be transposed as to predict the reaction in vivo. From the results of this study it was observed that the effects of the plant extracts in fresh milk were not significant. There was continuous increase in the lactic acid development over a period of time and eventually after 20 hours, milk curds were observed. Some treated milk samples exhibited even higher lactic acid percentage than the control though the difference was not statistically significant. These results agree with those obtained by Daramola [34] showing that the fermentation profile of milk can be altered when such milk is supplemented by plant extracts. In their study, the authors used biberry (*Vaccinium myrthillus*) and liquorice (*Glycyrrhiza glabra*) extracts, both plant extracts enhanced fermentation process as shown by increase in lactic acid of the extract added samples in comparison to the sample without plant extract.

Furthermore, increasing the concentration of the extracts showed no added antimicrobial effect. However, it resulted in the change of the color of the milk from cream to different colors depending on the color of the extract. The extrapolation of results obtained from in-vitro experiments with laboratory media to food products is not straightforward as foods are complex, multicomponent systems consisting of different interconnecting microenvironments. The presence of protein, carbohydrates and fat in actual food decreases the antimicrobial effect of the herbs [35]. The greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster [36]. Hence, the level of preservatives required for sufficient efficacy may be considerably higher in food products in comparison with laboratory media, which may negatively impact the organoleptic properties of food [8]. Hence more experimental studies must be done to able to effectively determine the appropriate concentrations that can be added to the milk while putting into consideration the taste and color of the milk. As opposed to the study that utilized plant extracts, the women in the pastoral communities utilize only the smoke from the plant species.

5.0 Conclusion and Recommendations

5.1 Conclusion

In conclusion, this study established that in Kilosa district's Mbwade and Parakuyo pastoral villages, the women use the same technique to impart flavor to the milk and reduce milk degradation. The technology involved smoking of the insides of the milk storage containers using dry plant stem cuttings, prior to addition of fresh milk. The stems of *D. melanoxylon* exhibited greater activity compared to the other extracts and *Salmonella typhi* was the most susceptible organism across extracts. When preservative effects of the extracts were tested in fresh milk, the extracts did not show significant effect in reducing the development of lactic acid than the untreated milk. Increasing the concentration of the plant extracts did not improve the antimicrobial effect in milk. The antimicrobial properties of these plants may be of great use for the development of both plant antimicrobials and natural food preservatives and inclusion of indigenous knowledge is imperative in solving local problems such as absence of milk refrigeration systems in rural areas, and milk intolerance.

5.2 Recommendations

Though there is rich ethnobotanical indigenous knowledge among the Maasai pastoral women of Mbwade and Parakuyo villages in Kilosa district, the women, do not earn income from such because their innovations and practices are mostly organized, accumulated and embedded in a context through experience, and these indigenous technologies are applied in isolation. Therefore, to salvage and record this indigenous knowledge, various community biogical-prospecting studies must be conducted to search and record the local uses of various plant species. This can be a useful tool in the designing and development of a viable and sustainable local milk preservation method, with an ultimate goal of improved pastoral food security and safety.

Further research must be done to assess milk quality between traditionally treated milk and non-treated using microbiological milk parameters of bacterial cell count. Clinical trials should be carried to ascertain the claim of the efficacy of treated milk against milk intolerance.

Acknowledgements

Acknowledgements to the pastoral women in Kilosa District for sharing their knowledge, many thanks to Dr Yuda Mgeni the District Veterinary officer, Mr. Ismail Almas, Maurice Mbunde and Abdul Waziri of Muhimbili University of Health Sciences and Allied Sciences- Institute of traditional medicine and Musa Mtuga of Sokoine University of Agriculture.

Funding

The financial support was provided by the Intra-African Caribbean and Pacific (INTRA-ACP) Mobility Scheme EACEA Project.

Availability of data and materials

The data that support the findings of this study are available from authors upon request.

Authors' contribution

All authors are responsible for all parts of this paper. They all designed the study. CK conducted the field work, laboratory data collection and drafting of the manuscript. HA, GB and RM contributed to the supervision and critical revision of the manuscript. All authors read and approved the final manuscript.

Ethical Approval and consent to participate

For ethical consideration a research permit was obtained from the Vice Chancellor Sokoine University of Agriculture. The purpose of the research was explained to the village leaders after which verbal consents were obtained. At household level, consent was obtained through self-introduction and explanation of the purpose of the study.

Competing interests

All authors declare that they have no competing interests.

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