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Antimicrobial Activities of Neem Extract (*Azadirachta indica*) Against Microbial Pathogens of Animal Origin

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Abstract: The antimicrobial activity of neem extract at different concentrations was evaluated using agar-well diffusion method against 13 microbial pathogens strains of animal origin. Results revealed that the neem extract has great bactericidal activities at lower concentrations 10 and 50% than at concentrations above 75 to 100%. Diluted neem extract showed bactericidal activities against Gram negative bacteria but did not against Gram positive bacteria. Zone of inhibition reached 14 and 12 mm against *Citrobacter,* 19 and 18 mm against *Klebsiella,* 18 and 17 mm against *S. bodyi,* 18 and 15 mm against *S. sonnei,* 13 and 12mm against *S. flexeneri,* 14 and 12 mm against *E. coli* O157, 17 and 15 mm against *E. coli* O78*,* 14 and 13 mm against *E. coli* O26 and16 and 14 mm against *Salmonella* at conc. 10 and 50%, respectively. Neem extract has no antibacterial activities against tested Gram positive bacteria; *S. aureus* and MRSA. Against mycotic isolates only 10 % of Neem extract showed fungicidal effect with zone of inhibition 25 and 20 mm against *C. albicans* and *Asp. flavus*, respectively. Neem extract was evaluated for its capability for hindrance of bacterial count in ground beef as well as monitoring of its capability for hindrance of *E. coli* O157 ATCC 700728 inoculated in ground meat. Neem extract significantly decreases bacterial count Mean± SD from 1.90±0.35 to 0.0064±0.0002cfu/ml before and after addition of neem, respectively. Inoculated of ground meat with *E. coli* O157 ATCC 700728 in relation to addition of 10% neem extract, showed significant decline of aerobic bacterial count Mean± SD from 78.00±2.31cfu/ml to 0.0310±0.0015cfu/ml and *E. coli* count from 0.60±0.23 to 0.0012±0.0002, respectively. Results concluded that, diluted neem extract showed great antimicrobial properties at low concentration (10%) with significant decrease of bacterial count after addition of 10% neem extract. It is recommended that further work should be done to identify the specific ingredient(s) responsible for this effect, purify it and standardize as a preservative against microbial contaminant of food from animal origin.

Key words: Neem · Antimicrobial · Agar well diffusion method · *E. coli* O157 · Bacterial count · Ground beef.

INTRODUCTION

Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. According to reports of the World Health Organization, 80% of the world's population relies mainly on traditional therapies, which involve the use of plant extracts or their active substances [1].

Microorganisms have developed resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs [2]. Furthermore, antibiotics are

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sometimes associated with side effects [3], whereas there are some advantages of using antimicrobial compounds of medicinal plants, such as fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature [4].

The tree, *Azadirachta indica* of the family *Maliaceae;* popularly known as neem tree, has been in use since ancient times, to treat a number of human ailments and also as household pesticide [5-7]. Neem was used in traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries. Some studies on neem have showed that it contains active substances with multiple medicinal properties [8, 9]. The chemical composition of neem has been identified and the most active ingredient is reported as azadirachtin [10].

Neem leaves previously reported to have antibacterial properties and could be used to control airborne bacterial contamination [11]. This supported by the use of neem seeds in traditional medicine to treat infections [12-14]. Recently, *in vitro* study has demonstrated that aqueous extract of neem leaves prevent biofilm formation and adhesion in composite resin by *C. albicans* [15]. Moreover, the methanolic extract of neem was reported to have *in vitro* antimicrobial activities against *S. aureus, E. coli, Ps. aeruginosa* and *C. albicans.*

The purpose of the present study was to investigate the antimicrobial activity of neem plant leaves against pathogenic bacteria of animal origin, including Gram negative bacteria; *Citrobacter, Klebsiella, S. bodyi, S. sonnei, S. flexeneri, E. coli* O157, *E. coli* O78, *E. coli* O26 and *Salmonella* Typhimurium as well as Gram positive bacteria; *S. aureus* and MRSA as well as mycotic strains; *C. albicans* and *Asp. flavus.* Besides, the present study through light on the ability of neem extract to decrease the microbial contaminants in ground meat with special reference to *E. coli* O157ATCC 700728.

MATERIALS AND METHODS

Selection of Plant: The plant neem (*Azadirachta indica*) was selected for study. Its leaves were collected and identified from the market.

Leaf Extracts Preparation [16].

Preparation of Methanol Extract: Fresh neem leaves were collected and the leaf was washed, chopped and blended. Afterwards, the completely shade dried material was coarsely powdered. Fifty grams of dried leaf powder were taken in a separate container, then 250 ml of methanol was added and kept for 24 h with periodic shaking then filtered and the filtrate was collected. The procedure was repeated three times with fresh volume of methanol. The filtrates were pooled.

Microorganism: Thirteen completely identified pathogenic isolates of animal origin were used for evaluation of antimicrobial activities of neem extract *(Azadirachta indica)* at different concentrations. Isolates include; Gram negative bacteria; *Citrobacter, Klebsiella, S. bodyi, S. sonnei, S. flexeneri, E. coli* O157, *E. coli* O78, *E. coli O26* and *Salmonella* Typhimurium as well as Gram positive bacteria; *S. aureus* and MRSA as along with mycotic isolates; *C. albicans* and *Asp. flavus*.

Antimicrobial Screening

Agar Well Diffusion Method [17].

Medium: Muller Hinton Agar plates were prepared with a uniform thickness of approximately 4mm and agar is allowed to set at ambient temperature to solidify.

Inoculums: Bacterial and mycotic strains were inoculated in peptone medium and incubated at 37°C and 28°C respectively for 24 h and then serial dilutions were carried out to match 0.5 McFarland which was used as inoculums.

Method: The antimicrobial activity of neem extract was evaluated against bacterial and mycotic isolates using agar-well diffusion method. Neem at different concentrations; 500, 750, 900, 1000, 100 µl/ml DMSO were tested for their antimicrobial activities against 13 isolates. Hundred microliters of cell culture suspension matching with 0.5 McFarland of target isolate was spread onto the plates. For the investigation of the antibacterial and antimycotic activity, 100 µl of tested neem at different concentrations were added into wells of agar plates directly. Plates were t for 1 h at 25°C to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were re-incubated at 37°C and 28°C for 24 h for bacterial and mycotic isolates, respectively. After incubation, plates were observed for antimicrobial activities by determining the diameters of the zones of inhibition for each of the samples figures (1-6). For an accurate analysis, tests were run in triplicate for each isolate to avoid any error.

Experimental Evaluation of Neem Extract on Bacterial Count in Ground Beef: Ground beef was divided into four equal weights of 25 g, samples were weighted in sterile polystyrene bags, then mixed in stomacher after addition

Table 1: Antimicrobial activities of neem extract at different concentrations against tested bacterial and mycotic isolates of animal origin.

Neem extract conc.					
Bacterial isolates	10%	50%	75%	90%	100%
Citrobacter	14	12	11	-	-
Klebsiella	19	18	16	15	-
S.bodyi	18	17	-	15	-
S. Sonnei	18	15	16	-	-
S. flexeneri	13-16s	12-15s	13s	-	-
E.coli O157	14	12	-	-	-
E.coli O78	17	15	-	-	-
*E.coli*O26	14	13	-	14	-
Salmonella Typhimurium	16	14	-	-	-
S. aureus	-	10	-	-	-
MRSA	-	-	-	-	-
C. albicans	25	-	-	-	-
Asp. flavus	20	-	17s	-	-

Bactericidal effect show zone of complete microbial inhibition, shown in number Bacteriostatic zone was given as (s). (-) = negative results (no zone of inhibition)

Table 2: Total heterotrophic plate count of ground beef in relation to addition of 10% neem extract and inoculated with *E. coli* O157 ATCC 700728.

	APC without *E. coli* O157	APC with *E. coli* O157	EMB with *E. coli* O157	Sig.
Without Neem Extract	1.90±0.35[a]	78.00±2.31[b]	0.60±0.23[a]	0.0001
With 10 % Neem Extract	0.0064±0.0002[b]	0.0310±0.0015[c]	0.0012±0.0002[a]	0.0001

Means with different superscripts in the same column are significantly different at $p<0.05$

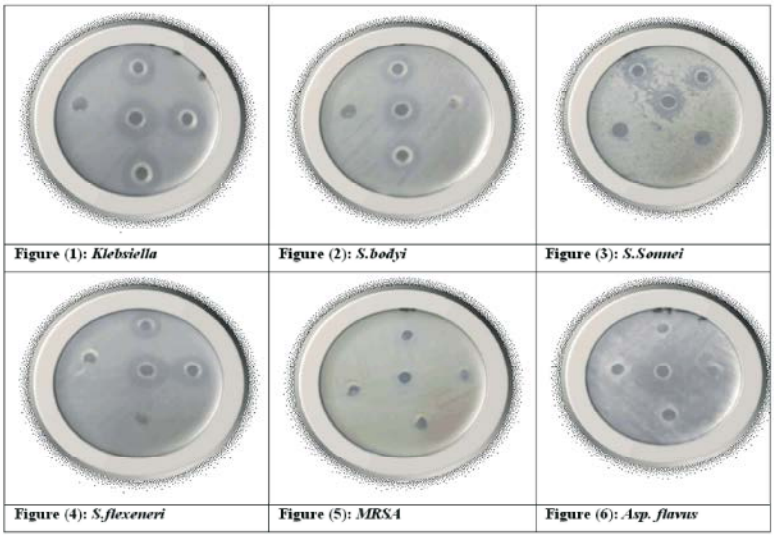

Fig 1. 1: *Klebsiella*, 2-*S.bodyi,* 3-*S.Sonnei,* 4-*S.flexeneri,* 5- *MRSA,* 6- *Asp. flavus.* (50, 75, 90, 100% from the top, clock wise and 10% in the center of the plates).

Fig 1-6: Showing the Antimicrobial Activities of Neem at different concentrations against the tested isolates.

of 225ml of buffered peptone water media. Neem extract was added into two samples to give final concentration of 10% and the other 2 samples were left without neem extract (control positive for bacterial growth without treatment). Then two samples (with and without neem) were inoculated with 1ml of 0.5 McFarland *E. coli* O157 ATCC 700728. Heterotrophic plate count (EN ISO 4833: 2002) [18] carried out using plate count agar and enumeration of *E. coli* (ISO FDIS 7218 (2007) [19] for both samples with and without neem extract inoculated and not inoculated with *E. coli* O157 ATCC 700728. Then bacterial count was compared in the four groups before and after addition of neem extract and inoculation of *E. coli* O157ATCC 700728.

Statistical Analysis: Simple one way ANOVA and independent sample t-test using SPSS (2007) [20] was used to study the effect of media without neem or with

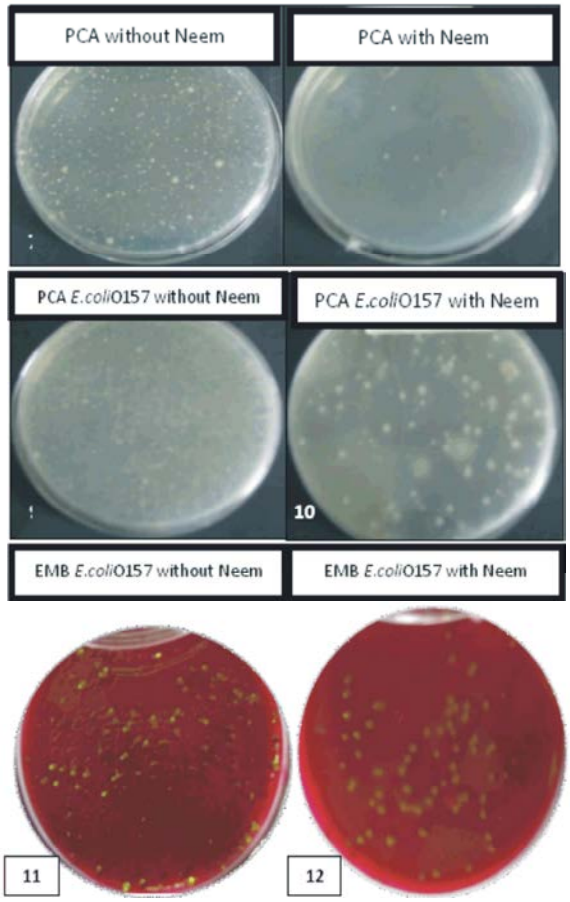

Fig 7-12: Bacterial count of ground beef in relation to addition of 10% neem extract and inoculation with *E. coli* O157 ATCC 700728.

10% neem on *E. coli* O157 ATCC 700728 count number. Data are presented as mean ± SEM. Duncan's multiple range tests was used to differentiate between significant means.

RESULTS

Results revealed that the neem methanolic extract has great bactericidal activities at lower concentrations 10 and 50% than at concentrated forms 75, 90 and100%. Also, results revealed that the neem diluted form has greater bactericidal effect on Gram negative bacteria than in Gram positive bacteria. Zone of inhibition reach 14 and 12mm against *Citrobacter,* 19 and 18mm against *Klebsiella,* 18 and 17mm against *S. bodyi,* 18 and 15mm against *S. sonnei,* 13 and 12mm against *S. flexeneri,* 14 and 12mm against *E. coli*O157 ATCC 700728, 17 and 15mm against *E. coli* O78, 14 and 13mm against *E. coli* O26 and16 and 14mm against *Salmonella* Typhimurium at concentrations 10 and 50%, respectively as shown in table (1) and figures (1-4).

Results also showed that neem has no bactericidal or bacteriostatic effect against tested Gram positive bacteria; *S. aureus* and MRSA as shown in table (1) and figure (5).

Against mycotic isolates only 10 % of neem extract showed fungicidal effect with zone of inhibition 25mm against *C. albicans* and 20mm against *Asp. flavus* as shown in table (1) and figure (6).

Experimental Evaluation of Neem Extract on Bacterial Count in Ground Beef: Neem extract was evaluated for its capability for hindrance of total bacterial count in ground beef as well as monitoring of its capability for hindrance of *E. coli* O157 ATCC 700728 after inoculation into ground meat. Heterotrophic plate count carried out onto PCA (plate count agar) as well as EMB (Eosin Methylene Blue agar) revealed a significant effect of neem extracts on bacterial count in ground beef with decline in the bacterial count (Mean±SD) from 1.90±0.35 to 0.0064±0.0002 cfu/ml before and after addition of neem, respectively (Table 2). Heterotrophic plate count as well as *E. coli* count using of ground beef inoculated with *E. coli* O157 ATCC 700728 with and without addition of 10% neem extract was enumerated using PCA (plate count agar) as well as EMB (Eosin Methylene Blue agar), results revealed a great decrease in the aerobic bacterial count as well as the *E. coli* count from Mean±SD of bacterial count from 78.00±2.31cfu/ml to 0.0310±0.0015 cfu/ml and with decrease in *E. coli* count from 0.60±0.23 cfu/ml to 0.0012±0.0002 respectively (Table 2) and figures (7-12).

DISCUSSION

Nowadays, the up growing resistance of microorganisms to the convectional antimicrobial agents is a source of great concern to clinical microbiologists. Bacteria evolve some changes in their genome with time, as a result, a large number of bacterial species particularly *Shigella* and *E. coli* [21] have become resistant to the antibacterial drugs due to extensive use and often create a problem in treatment of infectious diseases.

Medicinal plants having antimicrobial compounds in comparison with antibiotics, usually with fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature [22].

The results of the present work revealed that the leaves extract of neem showed an interesting inhibitory action on a wider spectrum of microorganism (11 out of 13 tested bacterial strains) using agar well diffusion technique and the resistant bacterial strains were *S. aureus* and MRSA. Neem extract has great bactericidal activities at lower concentrations 10 and 50% than at higher concentrations of 75, 90 and100%. On the other hand Chindo *et al*. [23] proved that neem oil showed inhibitory action against *Salmonella* Typhi although neem soap was completely inactive. They added that both oil and soap were active against *S. aureus* which disagree with the present study showing no inhibitory activities against *S. aureus* or MRSA.

Zone of inhibition reach 14 and 12 mm against *Citrobacter,* 19 and 18 mm against *Klebsiella,* 18 and 17 mm against *S. bodyi,* 18 and 15 mm against *S. sonnei,* 13 and 12 mm against *S. flexeneri,* 14 and 12 mm against *E. coli* O157 ATCC 700728, 17 and 15 mm against *E. coli* O78, 14 and 13mm against *E. coli* O26 and16 and 14 mm against *Salmonella* at concentrations 10 and 50%, respectively as shown in table (1) and figures (1-4). Results revealed that neem extract has no bactericidal or bacteriostatic effect against tested Gram positive bacteria; *S. aureus* and MRSA as shown in table (1) and figure (5).

It was reported that *E. coli* showed 15 and 14 mm zone of inhibition for ethanol and methanol extracts of neem, respectively [24]. It was confirmed that native extracts of neem leaves with concentration of 20 Cg/disc showed inhibitory activities against *S. aureus*, *E. coli, C. albicans, Asp. niger* and *Penicilium citrinum* [25]. Aqueous extract of the neem was reported for their anti-inflammatory, antimicrobial and immunomodulatory activities [26]. It was reported that ethanol extracts of Neem showed good inhibitory activity with low MIC concentration (75-250 Cg/ml). They confirmed that native extracts of neem leaves with concentration of 20 Cg/disc have inhibitory activities against *S. aureus, E. coli, C. albicans, Asp.niger* and *P.citrinum* [25].

Results partially agreed with other researcher who indicated that neem oil extracted from the leaves, seeds and bark, possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms, also it inhibits *Klebsiella pneumonia in vitro* [27]. The present study revealed that neem extract was effective against *Klebsiella* with zone of inhibition equals19 mm as shown in figure (1).

On the other hand, results totally disagree with the finding of Hoque *et al*. [28] who reported that extract of neem did not show antimicrobial activity against any of the Gram-negative bacteria tested but were highly effective in controlling Gram-positive and spoilage microorganism.

Against mycotic isolates only 10 % of neem extract showed fungicidal effect with zone of inhibition 25mm against *C. albicans* and 20mm against *Asp. flavus* Table (1) and figure (6). Results agreed with the findings of other researcher who found that leaf extracts of neem have interesting inhibitory action on a wider spectrum of microorganisms, including *C. albicans*, multi-drug resistant *Staphylococcus aureus*, urinary tract *E. coli* with safety of the used formulations and acceptability [29]. Also, other researcher found that 20 % aqueous neem leaf extract had a toxic effect on 19 out of 22 tested moulds including *Asp.flavus* [30].The different types of extracts from neem leaves were found to have inhibitory effect on *C. albicans* [31]. It was proved that the fungicidal and bactericidal properties of extracts from neem leaves either *in vitro* or *in vivo* trials to the presence of several antimicrobial active ingredients in leaves of neem tree such as desactylimbin, quercetin and sitosterol [32]. They found that during assay with aqueous and organic extracts from neem leaves, their inhibitory effect of all used concentrations against pathogenic fungi including four *Aspergillus* species (*A. niger, A. flavus, A. terries* and *A. fumigatus*), in addition to *Microsporum gypseum* and *Candida albicans*. All concentrations of the aqueous extract effectively suppressed the mycelial growth of these fungi and this effect was found to increase with concentration where a maximum activity was reached using the last one (20%). It was found that 20 % aqueous neem leaf extract had a toxic effect on 19 out of 22 tested moulds including *Asp. flavus* [30]. Also, it was found that different types of extracts from neem leaves were found to have inhibitory effect on *C. albicans* [31].

It was stated that neem oil which showed considerably activity against Gram-positive bacteria; *Staphylococcus* species and Gram-negative bacteria; *E. coli, B, cereus, P. vulgaris, S.*Typhi, *Klebsiella pneumonae, S. dysenterae* and fungal strain; *Fusarium oxysporum, Asp. flavus, Asp. fumigates, Asp. niger, C.albicans* [33].

The antifungal activity of neem oil against above fungal strains showed considerably activity. Moreover, the aqueous extract of plant has been previously reported to show antifungal activity [25, 34].The previous findings agreed with that of the present work which proved the fungicidal activities of the neem extract against *C. albicans* and *Asp. flavus* as shown in table (1) and figure (6).

Results were confirmed with evaluation of neem extract its capability for hindrance of total bacterial count in ground beef as well as monitoring of its capability for hindrance of *E. coli* O157 ATCC 700728 after inoculation into ground meat. Heterotrophic plate count revealed a significant effect of neem extracts on bacterial count in ground beef with (Mean ± SD) bacterial count reach 1.90±0.35 before using neem extract which was reduced to 0.0064±0.0002 cfu/ml after addition of 10% neem, as shown in table (2). Heterotrophic plate count of ground beef inoculated with *E. coli* O157 ATCC 700728 with and without addition of 10% neem extract was decreased significantly from Mean±SD bacterial count of 78.00±2.31cfu/ml to 0.0310±0.0015 cfu/ml, respectively. The enumeration of *E. coli* onto EMB decreased from 0.60±0.23 to 0.0012±0.0002 (Table 2) and figures (7-12). These finding confirm the great antibacterial effect of 10% neem extract when added to ground meat.

CONCLUSION

In this study, neem extracts showed antibacterial activity against selected foodborne pathogens and spoilage microorganisms. The result of this study also suggests that neem extracts possess compounds containing antibacterial properties that can be useful to control foodborne pathogens and spoilage organisms due to significantly decrease of bacterial count after addition of 10% neem extract. Antibacterial and antifungal activities of neem extract obtained in the present study will be applied to actual foods to assess the microbiological condition of the particular food or food products with extended shelf-life.

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