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Inhibitory effect of garlic extract on multidrug resistant *Salmonella* isolated from broiler chicken meat

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Abstract
A survey was conducted to screen the presences of antibiotic-resistant *Salmonella* from broiler chicken meat brought from local retail shops situated at Kozhinjampara, Palakkad, Kerala. Based on the morphological and biochemical characterization, a total of 15 *Salmonella* sp were isolated. Among 15 isolates, five (S7, S8, S10, S12, S14) isolates exhibited 100% resistance to all tested antibiotics and only one isolate S9 shows sensitivity to maximum antibiotics tested. In the study, garlic extract was tested against antibiotic-resistant isolates and it exhibited maximum inhibition against S12 isolate (30mm) and minimum inhibition against S8 isolate (22mm). The analyses of the garlic extract by GC-MS confirmed that the major components of garlic having antimicrobial activity. The result specified that garlic extract was found to be effective in controlling antibiotic resistance *Salmonella* species from broiler chicken meat. Therefore, incorporating garlic products in poultry feeds will help reduce antibiotic-resistant pathogenic microbes which causes drastic infections in human beings.

Keywords: antibiotics, garlic, multidrug-resistant, poultry, *Salmonella* sp

1. Introduction

Nowadays, over 90 billion tons of chickens are commonly farmed for consumption per year (FAO, 2017). During the last three decades, the poultry industry has seen unparalleled growth and also recognized as one of the fastest-growing agricultural sectors. Because, increased consumption of meat and eggs is due to its vital nutrients such as dietary minerals, vitamins etc in an affordable cost [1].

Salmonellosis is one of the major zoonosis disease caused by the consumption of animal origin especially poultry products. Around 70–80% of foodborne illness are associated with the uptake of *Salmonella* contaminated livestock and poultry products in various countries. More than 2,600 *Salmonella* serovars were identified worldwide and almost all serovars cause illness in humans and also in animals. The important reason for salmonellosis was the unhygienic handling of raw poultry meat and the ingestion of undercooked poultry products [2, 4]. To improve the growth performance of broiler chickens, antibiotic growth promoters have been added to feed additives to improve performance efficiency and to prevent disease in poultries [5]. However, the continued overuse of antibiotics in poultries causes antibiotic resistance in *Salmonella*, which is one of the utmost public health problems suffering worldwide. The presences of multidrug-resistant (MDR) *Salmonella* from antibiotic administrated chickens could pose a serious threat to humans through the food chain.

A large number of antibiotics and hormones are used to grow poultries in many countries as a business motif. The overuse of such antimicrobials in poultry production probably enhances the development of antibiotic resistance in pathogenic microbes as well as in normal commensal microorganisms. In addition, there are also human health concerns about the presence of antimicrobial residues in meat, eggs, and other animal products. Several antibiotics such as penicillin, tetracycline, macrolide, aminoglycoside and amphenicol have been detected in foods. Antibiotic resistance is a worldwide phenomenon resulting in the occurrence of pathogens with resistance to clinically important antibiotics, demanding new treatment approaches. Antibiotic-resistant bacteria is the major reason for life-threatening illness and leads to a significant threat to the health and well-being of human beings. It is estimated that antibiotic-resistant pathogens cause ~2 million illnesses and 23,000 deaths annually in the U.S [6].

Foodborne pathogens such as *Salmonella* exhibit antibiotic resistance, which is a major concern for public health safety. More focus is required to target them in the animal foods supply [7]. The non-typhoidal *Salmonella* causes the highest number of illnesses, hospitalizations, and deaths associated with foodborne illness [8]. It is associated with more than 1,200,000 illnesses annually, and among these at least 100,000 infections are due to antibiotic-resistant *Salmonella*. Most of them are resistant to clinically important drugs such as ceftriaxone (36,000 illnesses/year) and ciprofloxacin (33,000 illnesses/year). In fact, *Salmonella* isolates conferring resistance to ≥5 antibiotics accounted for more than 66,000 illnesses from 2009 to 2011 in the U.S (Jones, 1983). Because of the emergence of antibiotic resistance in microbes, the European Commission (EC) in 2006 banned the usage of Antibiotic growth promoters (AGP) in animal production (EC Regulation No. 1831/2003; http://eur-lex.europa.eu/en/index.htm).

Therefore, it is essential to search for natural alternatives to antibiotics and chemicals used in poultry production.

Consumers' pressure and worries about the harmful effects of antibiotic use and the ban of antibiotics in the EU have prompted researchers to think about alternatives to antibiotics [9]. The excessive use of antibiotics in conventional farms has endorsed resistance in *Salmonella* sp. The various researches reported that, when a conventional farm was converted to an organic farm, the prevalence of antibiotic-resistant *Salmonella* was reduced. Many research has been looking for natural agents with similar beneficial effects to chemical growth promoters. There is several of non-therapeutic substitutes that can alternate antibiotics use. Among these, the most popular are prebiotics as an alternative. Nowadays natural antimicrobials from plants have been used as the substitute for conventional antibiotic-resistance.

Garlic (*Allium sativum*) has been used for centuries because of its health benefits. The garlic and garlic products have shown a broad antibiotic spectrum against both gram-positive and gram-negative bacteria [10]. In addition, they are effective against many common pathogenic intestinal bacteria responsible for diarrhoea in humans and animals [11]. It was reported that the garlic derived products have been active against antibiotic resistant strains; in particular, garlic extract and allicin, an effective compound present in garlic have been shown bacteriostatic effects on various vancomycin-resistant Enterococci [10].

The antibacterial activity of garlic is widely attributed to its major thiosulfinate, allicin [12]. In a previous study, Ross *et al.* [13] have proved that garlic derivatives inhibit the growth of many enteric bacteria, including pathogenic bacteria. Indeed, allicin and garlic extracts have exhibited a wide spectrum of antibacterial activity, including *Escherichia* sp., *Salmonella* sp., and *Clostridium* sp. [14]. So in the current study garlic extract is tested against antibiotic-resistant *Salmonella* isolated from poultry meat and studied its bioactive components using GC-MS analysis.

Materials and methods

Sample collection

The broiler chicken meat samples were collected randomly from local shops situated in Kozhinzampara, Palakkad district, Kerala during the period of August 2018. A total of five samples have been collected and aseptically placed it in sterile plastic bags and then brought to the laboratory using an icebox.

Isolation of *Salmonella* from broiler chicken meat

Fresh broiler chicken meat samples were chopped finely and pre-enriched in buffered peptone water in screw cap bottles and incubated at 35 °C for 24 hours. One ml peptone water (pre-enriched with meat) was transferred to selective enrichment media commonly used for *Salmonella* is Rappaport Vassiliadis broth (RV broth) and incubated at 35 °C for 24 hours. After selective enrichment, a loopful of culture from enrichment broth were streaked onto Hektoen Enteric (HE) Agar and incubated at 35 °C for 24 to 48 hours. The plates were observed for the typical colonies of *Salmonella* having blue-green with black centred colonies and confirmed the isolated colonies with *Salmonella* Shigella (SS) agar showing colourless with black centred colonies were selected, pure cultured and stored at 4° C for further studies.

Screening for multi-drug resistant isolates

An antibiogram of selected isolates was determined by using the Kirby-Bauer disk diffusion method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) [15]. Pre-incubated 24 hour cultures of all the selected isolates were spread over Mueller-Hinton Agar (MHA) medium. The selected antibiotic discs such as ampicillin, penicillin, gentamicin, streptomycin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin were placed over the lawn and incubated at 35 °C for 18–24 hour. The zone of inhibition around each antibiotic disc was measured and recorded.

Morphological and Biochemical Identification of Multi-Drug Resistant (MDR) *Salmonella* Isolates

The selected MDR isolates were identified up to genera level based on the following growth characteristics, staining reactions and biochemical tests, which includes Indole production, Methyl red test, Vogus prosauker test, Citrate, Urease, Nitrate reductase, Triple sugar iron agar test, oxidase, catalase and Carbohydrate utilization test. The cultures matching the typical reactions of *Salmonella* described in Bergey's Manual of Determinative Bacteriology was used for further study.

Screening of Anti *Salmonella* Activity Using Crude Extracts of Garlic

Garlic (*Allium sativum*) extract preparation

Fresh garlic (*Allium ativum*) bulbs were used for the study. A hundred grams of garlic bulbs were peeled, weighed and surface sterilized using 70% ethanol. The 70% ethanol used for surface sterilization of garlic was allowed to evaporate in aseptic condition and it was homogenized using a sterile mortar and pestle. The homogenized mixture was filtered through Whatman no 1 filter paper and stored for further studies.

Antimicrobial Activity Using Agar Well Diffusion Assay

The multidrug-resistant isolates selected for the study were inoculated into 10 ml of sterile nutrient broth and incubated at 35 °C for 8 hours. The cultures grown in nutrient broth were swabbed on the surface of sterile Muller Hinton Agar (MHA) plates using a sterile cotton swab. Agar wells were prepared with the help of a sterilized well cutter with 10 mm diameter [16] and 100µl of garlic extract was added to the wells in the agar plates. The plates were incubated in an upright position at 35° C for 24 hours and the diameter of inhibition zones was measured.

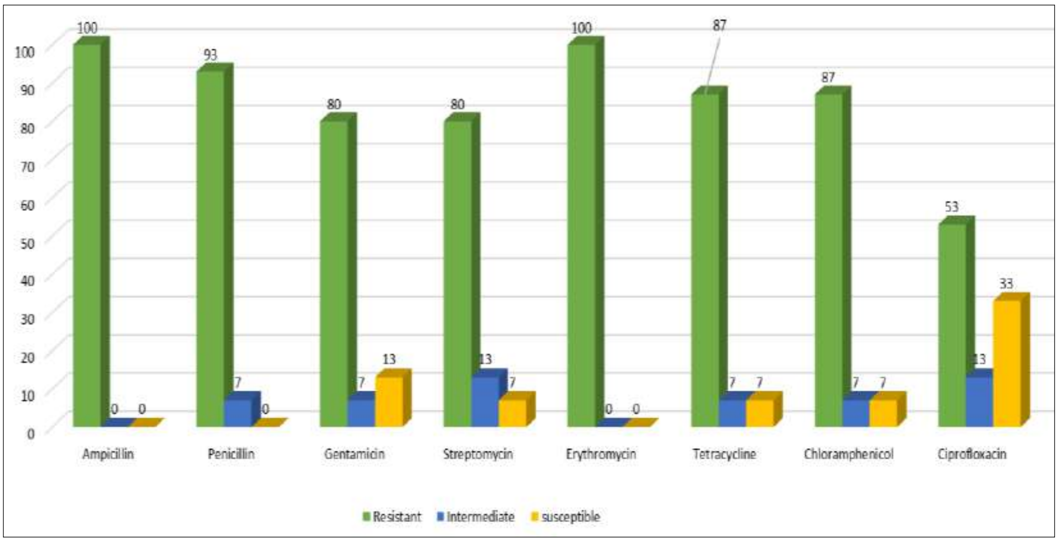
GC-MS analysis of garlic extract

The fresh garlic powder was subjected to GC-MS analysis. GC- equipped with a DB35 -MS fused silica capillary column (30 m length×0.25 mm outside diameter × 0.25 µm internal diameter) and GC interfaced to a Mass Selective Detector (MS-DSQ-II) with XCALIBUR software. An electron ionization system with an ionization energy of -70eV was used for GC MS analysis. Helium gas was used as a carrier gas at a constant flow rate of 1 ml/min and the sample injected was 2 µl; injector temperature was 250°C; ion source temperature was 200°C. The oven temperature was programmed from 70°C to 200°C at the rate of 6°C/min, held isothermal for 1 minute and finally raised to 260°C at 6°C/min. The comparative percent of the extract constituents was expressed as a percent with peak area normalization. The unknown compounds were compared with mass spectrum of the known compounds stored in the NIST library. The name and molecular weight of the components present in samples were ascertained [17].

Result and Discussion

Among five samples analysed 15 colonies were isolated based upon their cultural morphology. The result agrees with Bailey *et al*. [18] they found that broilers, which are in close proximity to the hatchery cabinet, can be easily cross-contaminated even if one single contaminated egg is present. According to Cox *et al.* [19] very few numbers of *Salmonella thyphimurium* from the excreta of infected chickens is sufficient to infect health ones. Yang *et al.* [20] reported that unhygienic practice of outlets, which were not properly monitored by appropriate authorities leads to the contribution of a high frequency of a number of *Salmonella* sp. Khan *et al.* [21] concluded a higher risk of *Salmonella* contamination in cottage poultry processors, than those sold at the hypermarket. Heyndrickx *et al.*, [22] reported that the main source of foodborne *Salmonella* enter into the farm from different sources, such as litter, water, equipment, vehicles, insects, rodents, and pets.

An antibiogram of isolated colonies was determined by using the Kirby-Bauer disk diffusion method. The result of antibiotic sensitivity test is shown in Figure 1. *Salmonella* isolated from broiler meat was resistant to Ampicillin (100%), Penicillin (93%), Gentamicin (80%), *Streptomycin* (80%), Erythromycin (100%), Tetracycline (87%), Chloramphenicol (87%), Ciprofloxacin (53%) and Intermediate to Penicillin (7%), Gentamicin (7%), *Streptomycin* (13%), Tetracycline (7%), Chloramphenicol (7%), Ciprofloxacin (13%) and sensitive to Gentamicin (13%) and *Streptomycin* (7%), Tetracycline (7%), Chloramphenicol (7%), and Ciprofloxacin (33%). From 15 isolates, five isolates (S7, S8, S10, S12, S14) exhibited 100% resistance to all tested antibiotics and only one isolate S9 shows sensitivity to maximum antibiotics tested. All isolates exhibited different antibiotic resistant pattern it may be due to the presences of different serovars of *Salmonella.* As similar to our study Jayaweera *et al.* [23] isolated antibiotic resistant *Salmonella* from broiler chicken in Sri Lanka and confirmed its presences in most of the samples they analysed. These results were in accordance with Salehi *et al.* [24], they isolated *Salmonella* from intestine and liver of broiler chicken, among them most of the isolates exhibited antibiotic resistant against trimethoprim, nalidixic acid, flumequine, tetracycline, streptomycin, kanamycin, amikacin and neomycin. Molla *et al.* [25] described that the commonly used antibiotics were found to be resistant against *Salmonaella* sp in veterinary and also in human health. *Salmonella,* most commonly carries plasmids, which encodes for antibiotic-resistance. Fluit *et al*. [26] described reduced uptake or expression of porins and changes in the cell, which cause reduced uptake or expression of efflux pumps leads to multidrug resistant in *Salmonella.* Most of the foodborne illness is caused by non-typhoidal *Salmonella* which causes the highest number of hospitalizations, and deaths [8]. Liljebjelke *et al.* [27] isolated multiple drug-resistant *Salmonella*, including gentamicin (12.6%), tetracycline (13.9%), sulfadimethoxine (20.9%), streptomycin (30.9%), and combination of trimethoprim-sulfamethoxazole (8.6%) were isolated from broiler chicken farms. Lorenz and Wackernagel, [28] reported the reason for antibiotic resistance in *Salmonella* serovars due to resistance gene transfer through cell-to-cell contact.



Footnote: Percent of antibiotic resistance towards poultry *Salmonella* sp

Fig 1: Percentage of antibiotic susceptibility against different bacterial isolates

As similar to the current study Gunasegaran *et al.* [29] studied the prevalence of *Salmonella* in curry samples and it reveals antibiotic resistances to ampicillin, tetracycline and chloramphenicol (100%). Medeiros *et al.* [30] studied the presences of antibiotic-resistant strains of *Salmonella* serovars such as *S. enteritidis, S. infantis, S. typhimurium*, and *S. heidelberg* in broiler carcasses. In addition, *S. enteritidis* isolated from raw, frozen, and stuffed chicken entrees associated with multistate disease outbreaks and were resistant to ampicillin and tetracycline [7]. Abiala *et al.* [31] reported all the *Salmonella* isolates showed gross resistance to amoxicillin, erythromycin and cefuroxime. The observed high resistance against these antibiotics probably reflects the high usage of the drugs in the poultry fields.

The isolates showing 100% antibiotic resistances were identified based on culture, staining and biochemical characteristics (Table 1). Selected isolates produced turbid growth on nutrient broth and smooth white to greyish white colony on nutrient agar with peculiar fetid odour, and black centred colonies on SS agar and white small non-lactose colonies on Maconkey agar. Gram staining shows Gram-negative, small rod and arranged as single or paired. Among five basic sugars only Glucose, maltose and mannitol were fermented with the production of acid and gas but lactose and sucrose were not fermented by most of the isolates. The isolate S10 was found to be non-motile, but the remaining isolates were found to be highly motile. Sannat *et al.* [32] reported nonmotile *Salmonella* species, and he identified it as *Salmonella pullorum or S. gallinarum.* The cultural characteristics of the above five isolates were suggestive of *Salmonella* organisms. Fowl typhoid caused by *Salmonella enterica*, produces high mortality rates (up to 90%) in birds of all ages, thus causing heavy economic loss. Fowl typhoid has been reported from various parts of India including Kashmir, Haryana, Kerala, and Kolkata. The isolation of *Salmonella* sp serotypes from carcasses of chicken was found to be varied by countries some serotypes such as *S.enteritidis, S.infantis, S.newport, S. heidelberg, S. kentucky, S. derby* and *S. typhimurium* were commonly isolated from chickens and associated with human infection outbreaks [33].

The anti-*Salmonella* activity of garlic extract was carried out by the well diffusion method. It exhibited a maximum zone of inhibition against all tested antibiotic-resistant *Salmonella* isolates. The maximum activity was against S12 (30mm) and minimum was against S8 (22mm) (Figure 2). In the case of MDR strains, the garlic acetone extract was reported to have the highest activity against *E. coli* MDREC1 and *K. pneumonia* MDRKP2 with 24 and 20 mm zone of inhibition [34]. Salem *et al.* [35] demonstrated that repeated administration of garlic extract to baby chicks colonized with *S. typhimurium* decreases the deaths and significantly upsurge the body weight of chicks.

Table 1: Morphological and Biochemical identification of MDR *Salmonella* isolates

Biochemical characters	**Selected bacterial isolates**				
	S7	**S8**	**S10**	**S12**	**S14**
Gram staining	-ve rod	-ve rod	-ve rod	-ve rod	-ve rod
Motility	+ ve	+ ve	- ve	+ ve	+ ve
Indole	-	-	-	-	-
Methyl Red test	+	+	+	+	+
Voges Proskauer test	-	-	-	-	-
Citrate	+	+	+	-	-
Catalase	+	+	-	+	+
Oxidase	-	-	+	-	-
Urease	-	-	-	-	-
Triple sugar iron	AK/A Gas H_2S	AK/A Gas H_2S	AK/A Gas H_2S	AK/A Gas H_2S	AK/A Gas H_2S
Nitrate	+	+	+	+	+
Glucose	+ G	+ G	+ G	+ G	+ G
Sucrose	-	-	-	-	-
Mannose	+	+	-	+	+
Lactose	_	_	_	-	-
Mannitol	+	+	-	+	+

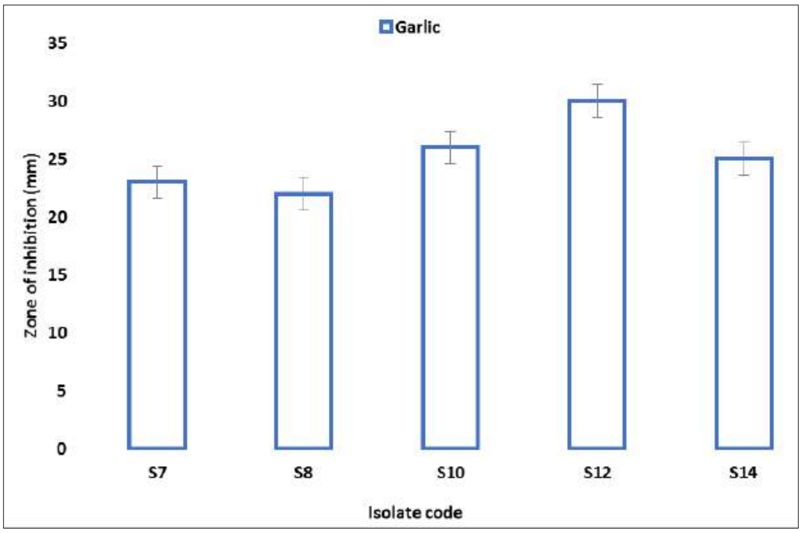


Fig 2: Anti-*Salmonella* activity of garlic extract

The garlic extract showing effective inhibition of MDR *Salmonella* sp was subjected to GC-MS analysis to know its bioactive components. A total number of 24 compounds were identified in garlic extract by comparing the GC-MS spectra with the NIST MS library. The emain components of garlic were (4-Bromophenyl) bis (2,4-dibromophenyl) amine (83.59), N-(-2-Acetamido)) -2-aminoethanesulfonic acid (40.20), 6,6''-Bis (chloromethyl) [4,4':6',4''-terdibenzofuran] (49.89), Dodecachloro-3,4-benzophenanthrene (63.04), 10-Methylcyclohexa [b] quinolin-1-one (40.95) (Figure 3 and Table 2). Various studies indicated that the sulfur containing volatile compound, allicin, is mainly responsible for the antibacterial effect of garlic, which limits the speed of RNA synthesis. Some other reports suggested that other volatile compounds of garlic such as diallylmonosulfide, diallyldisulfide, and diallyltrisulfide were also found to have antimicrobial properties [36, 37]. This plant contains carbohydrates, reducing sugars, lipids, flavonoids, ketones, alkaloids, steroids, and triterpenes. It is a remarkable plant, reported to have various pharmacological activities such as antimicrobial, antithrombotic, hypolipidemic, antiarthritic, hypoglycemic, and antitumor activity [38].

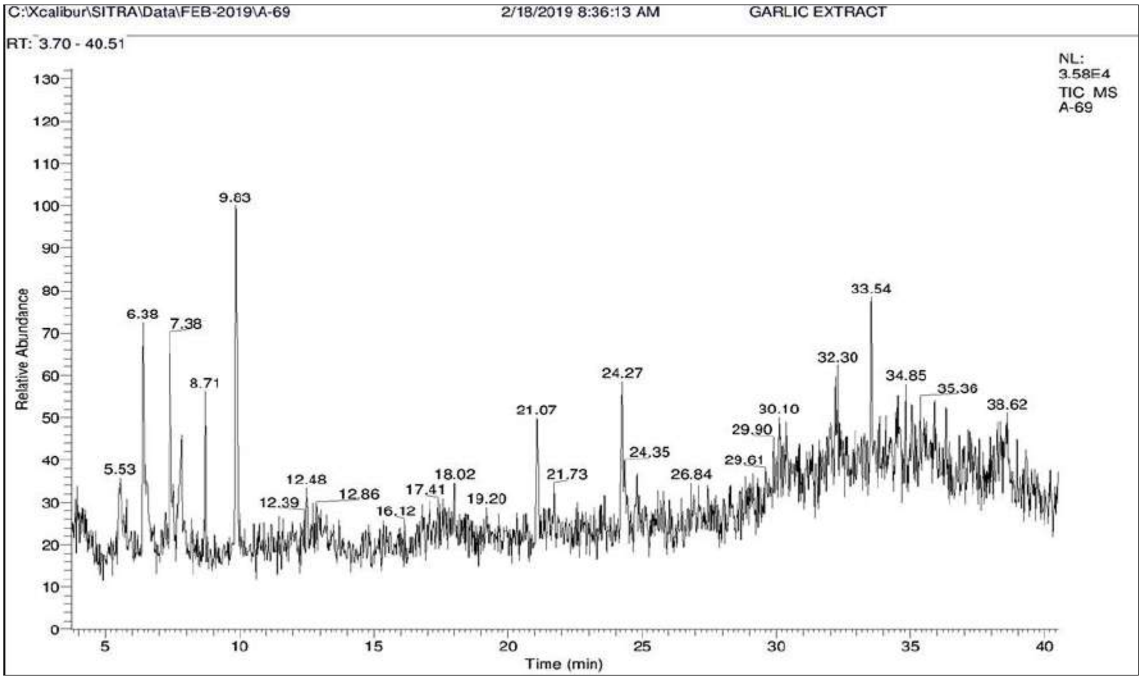


Fig 3: GC-MS analysis of garlic extract showing significant antibacterial activity against MDR *Salmonella*

Table 2: Bioactive compound profile of Garlic extract

Sl. No.	Compound name	Molecular formula	Molecular weight (gmol-1)	Probability	Area %	Retention Time
1	a-psi.-Carotene	$C_{4}0H_{56}O_{2}$	568	7.63	2.39	4.14
2	Docosane (CAS)	$C_{22}H4_{6}$	310	21.96	4.85	5.53
3	(N-(-2-Acetamido))-2-aminoethanesulfonic acid	$C_{4}H_{10}N2O_{4}S$	182	23.29	9.83	6.40
4	Furan, tetrahydro- (CAS)	$C_{4}H_{8}O$	72	27.79	6.77	7.40
5	(4-Bromophenyl)bis(2,4-dibromophenyl)amine	C18H10Br5N	635	83.59	5.26	7.81
6	1-O-Octyl-Glucitol	C14H30O6	294	6.96	3.54	8.71
7	(N-(-2-Acetamido))-2-aminoethanesulfonic acid	C4H10N2O4S	182	40.20	11.92	9.85
8	6,6''-Bis(chloromethyl)[4,4':6',4''-terdibenzofuran]	C38H22Cl2O3	596	49.89	1.97	2.48
9	2-Nonadecanone 2,4-dinitrophenylhydrazine	C25H42N4O4	462	9.73	1.96	12.96
10	3-Chloro-4-(formyloxy)tetrahydrofuran	C5H7ClO3	150	9.16	1.84	14.66
11	cis-2,4-Dimethylthiane	C7H14S	130	6.28	1.62	15.37
12	4-Hydroxymellein	C10H10O4	194	5.55	1.55	15.92
13	Dodecachloro-3,4-benzophenanthrene	C18Cl12	636	63.04	1.74	18.49
14	8-Azabicyclo[3.2.1]octan-3-one,	C8H13NO3	171	7.09	4.36	21.09
15	7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-	C8H12O2	140	5.45	6.18	24.27
16	Dodecane, 5,8-diethyl	C16H34	226	7.64	2.03	24.78
17	Isochiapin A	C19H26O6	350	14.61	3.40	25.76
18	cis-4,7,10,13,16,19-Docosahexaenoic acid,	C28H46O2Si	442	7.00	1.83	29.08
19	L-Glucose	C6H12O6	180	6.60	2.21	30.10
20	2-Trimethylsiloxy-6-hexadecenoic acid, methyl ester	C20H40O3Si	356	6.59	2.10	31.32
21	Dihydro-Terpineol	C10H20O	156	5.29	3.90	33.52
22	Oxiranedodecanoic acid, 3-octyl-, cis-	C22H42O3	354	5.06	1.55	34.26
23	Glycerine-1,3-dimyristate, 2-O-trimethylsilyl	C34H68O5Si	584	8.82	3.23	34.52
24	10-Methylcyclohexa[b]quinolin-1-one	C14H13NO	211	40.95	1.92	38.60

Conclusion and future scope

During last few decades, antibiotics have played a vital role in fighting against infectious diseases and also stimulants the growth of poultries. Scientific evidence suggests that their large-scale use has led to antibiotic resistance and residues in the food and the environment. Owing to the rise in consumer demand for livestock products from antibiotic-free production systems, there exists a great need for the development of antibiotic alternatives that can help improve performance and maintain optimal health of food animals.

These alternatives give equal or better effects to antibiotics (namely good livestock performance), reduce mortality rates and protect environment and consumer health. Recently much research has been diverted towards the search for antibiotic alternatives and which in turn has resulted in the enhanced use of probiotics, prebiotics herbal drugs, natural extracts etc. In the current study, the effect of prebiotic garlic shows maximum growth inhibition of *Salmonella* isolated from broilers. So, it can be effectively used to replace the antibiotic in poultry feed. Even though, there is an enormous quantity of research literature in this area, still there is an essential to found values of garlic use in poultry feeds. To fulfil this purpose more research is needed on these eco-friendly supplements.

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