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Capsaicin and gingerol analogues inhibit the growth of efflux-multidrug resistant bacteria and R-plasmids conjugal transfer

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**Abstract**

**Ethnopharmacological importance:** *Capsicum* and ginger are used widely in human diets and in folklore medicines. Chemically, gingerol is a relative of capsaicin and both classes of compounds are notable for their spiciness and characteristic pungent aroma. Previous studies have demonstrated that these compounds contain antimicrobial compounds with robust pharmacological importance.

**Aim:** The present study evaluated the in vitro antibacterial activities of capsaicinoids and gingerols against a panel of clinical MRSA strains and their inhibitory effect on the conjugal transfer of R-plasmids harboured in *E. coli*.

**Materials and methods:** Crude methanol extract of *C. annum* was fractionated using solid phase extraction (SPE) and screened for R-plasmid transfer inhibition: TP114, PUB 307, PKM 101, R6K and R7K. The bio-guided assay led to the isolation of bioactive compounds with strong R-plasmid transfer inhibition. The compounds were identified using Nuclear Magnetic resonance (NMR) and Mass spectroscopy (MS). Capsaicin analogues nonivamide, 6-gingerol, 6-shogaol, capsaicin and dihydrocapsaicin were screened for antimicrobial activity against a panel of methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria strains using microdilution method while the plasmid transfer inhibition assay of the compounds was determined by broth mating method.

**Results:** The bioactive fraction Ca-11 showed good inhibition rates (8.57-25.52%) against three R-plasmids PUB307, PKM 101, TP114 followed by the crude extract of *C. annum* (8.59%) respectively leading to the bioassay-guided isolation of capsaicin and dihydrocapsaicin as the bioactive principles. The antiplasmid effect of pure capsaicin and dihydrocapsaicin were broad and within active ranges (5.03 -31.76%) against the various antibiotic resistance-conferring plasmids including R6K, R7K. Capsaicin, 6-gingerol and 6-

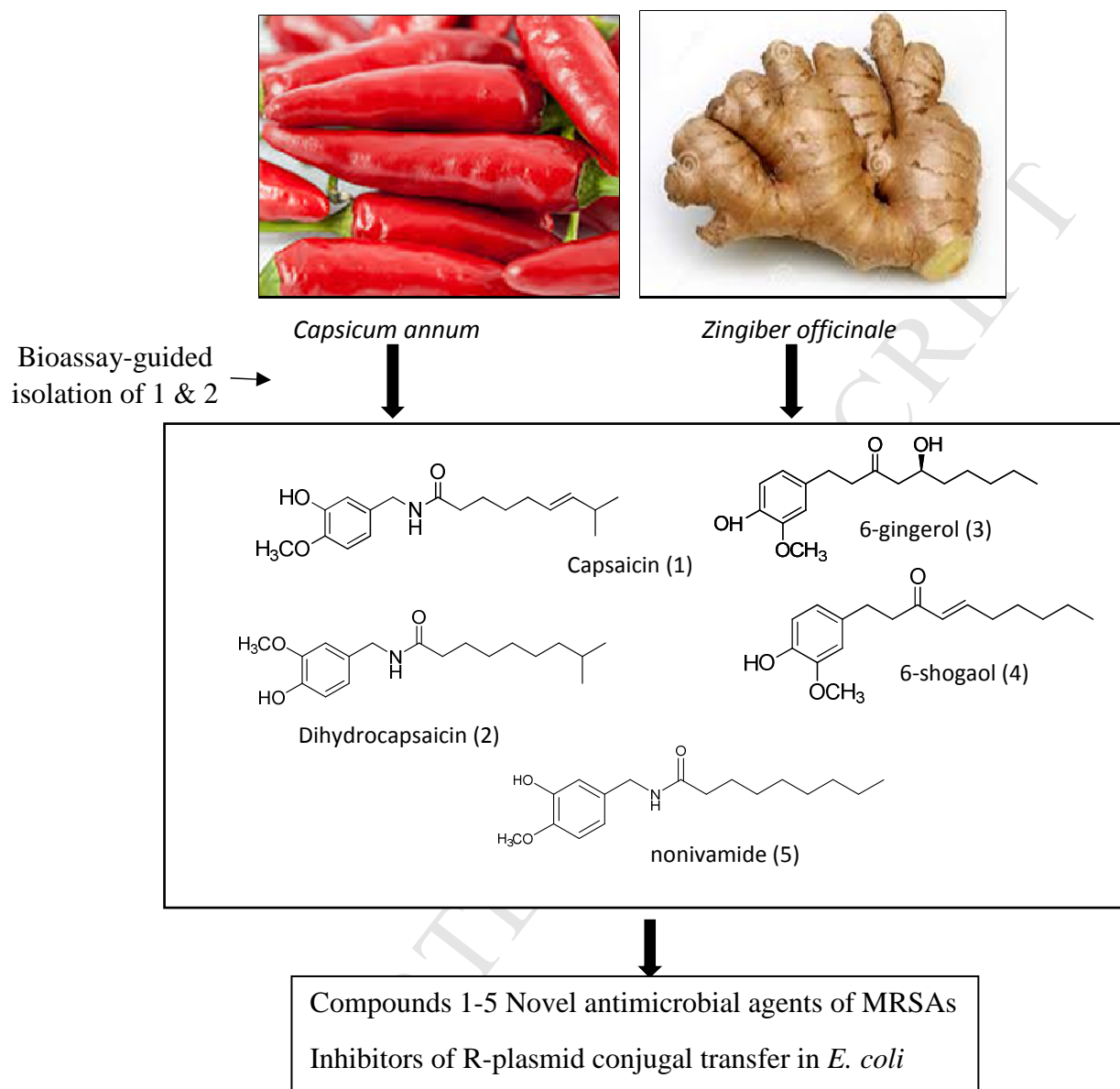
shogaol had good broad antibacterial activity with MIC values ranging from 8 to 256 mg/L against effluxing MRSA strains SA1199B (NorA), XU212 (TetK) and RN4220 (MsrA). While they exhibited moderate antibacterial activity (128-512 mg/L) against the Gram-negative bacteria. The effect of 6-gingerol, 6-shogaol and nonivamide on the plasmids were very active on PKM 101 (6.24 -22.16%), PUB 307 (1.22-45.63%) and TP114 (0.1-7.19%) comparative to the positive control plumbagin (5.70 -31.76%).

**Conclusion:** These results are suggestive that the R-plasmids could possess substrate for capsaicinoids-like compounds and for their ability to inhibit the plasmid conjugation processes. Plant natural products possess the potential value of antibacterial and mechanistic antiplasmid activity as demonstrated by the compounds and should be evaluated in developing antimicrobial leads to novel mechanism against multidrug resistant bacteria.

**Keywords:** Capsaicinoids, Gingerols, antimicrobial, multidrug resistance, Bacterial plasmids, MRSA

**Compounds studied in this article:** Capsaicin (PubChem CID: 2548), Dihydrocapsaicin (PubChem CID 71316141), Nonivamide (PubChem CID: 2998), 6-gingerol (PubChem CID: 442793), 6-shogaol (PubChem CID: 5281794)

## Graphical abstract



**Capsaicin and gingerol analogues inhibit the growth of efflux-multidrug resistant bacteria and R-plasmids conjugal transfer**

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Methicillin-resistant *Staphylococcus aureus* (MRSA), a prominent 'ESKAPE' pathogen continues to serve as reservoirs of multiple virulence determinants and pose difficulties to available antibiotic treatment. The global health implications of MRSA infections are daunting as 64% of infected patients are more likely to die compared patients infected with the non-resistant form of the pathogen (WHO 2017). The wrong use of several antibiotics among other factors over time has continued to drive the persistence of multidrug-resistant MRSA. Along with intrinsic mutations of target proteins and the presence of efflux genes, multidrug-resistant MRSA strains often acquire and transfer resistant plasmid (R-plasmid) facilitating antibiotic resistance transfer among intra- and inter-bacterial communities (Walsh 2016). Plasmid employs highly stable backbone which utilises type IV secretion systems (T4SSs) during horizontal transfer (Christie et al. 2014) and this target has gained a renewed interest recently as researchers seek to find novel targets and drugs to combat the supposedly resistant 'superbugs'.

Previous studies have identified antimicrobial agents that act on curing or inhibition of R-plasmid such as anionic and cationic surface-active compounds, ethidium bromide, novobiocin, (Spengler et al. 2006) but their use as an antimicrobial agent and therefore use as anti-plasmid agents were severely hampered by toxicity issues. Not to say the least, naturally derived compounds remain a potential source of new antibacterial agents such as plumbagin (Ding et al. 2005), rottlerin (Oyedemi et al. 2016), linoleic and dehydrocrepynic acids (Ferdanez-Lopez et al. 2005), previously reported for desirable antibacterial and anti-plasmid potentials.

Capsaicin constitutes the 90% abundance than other low abundance capsaicinoids such as dihydrocapsaicin, norcapsaicin, nordihydrocapsaicin, normordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin (Garces-Claver et al. 2007). Nonivamide is another naturally occurring analogue of capsaicin although first appeared in capsaicin- labelled products as an adulterant and thus regarded as synthetic capsaicin (Reily et al. 2001). The ethnopharmacological importance of Genus *Capsicum* and its main bioactive compound capsaicin date thousands of years to the tropical and humid zones of Central and South America (Zimmer et al. 2012). Peppers from *Capsicum annum* and other over 200 varieties are commonly used as a spice or food and for a broad range of therapeutic applications in Indian, Native American and Chinese medicinal traditions for the treatment of arthritis, rheumatism, stomach ache, skin rashes, dog/snake bite and wounds (Meghvansi et al. 2010). In West Africa, particularly among the Igbo culture, hot pepper soups is a local relish rich in high doses of chilli pepper and combination of various spices is highly recommended for morning sickness and vomiting, restores gastric tone and promotes wholesome digestion of food. Several studies continue to show the increasing use of capsaicin in pain management and many pharmaceutical applications (Derry et al. 2017, Evangelista 2015). Capsaicin serves as antioxidants, antidiabetic, anti-inflammatory, and contained in commercial drugs, agricultural and food products (Jolayemi and Oyewole 2013, Srinivasan 2016). Studies demonstrated the antimicrobial effect of capsaicin against pathogenic *E. coli*, *Pseudomonas solanacearum*, and *Bacillus subtilis* (Noumedem et al. 2013), metronidazole-resistant *Helicobacter pylori* (Zeyrek Yildiz and Oguz, 2005) and the inhibition efflux pump NorA (Kalia et al. 2012).

Gingerol commonly known as 6-gingerol is the active pungent agent in plant *Zingiber officinale* Roscoe belonging to the family Zingiberaceae, popularly called ginger based on (<http://www.plantlist.org>). Other constituents of ginger, include gingerol include [4]-, [8]-,

[10]-, [12]-gingerol collectively known as gingerol, [6]-, [8]-, [10]-shogaol, and essential oils (Schweiggert et al. 2008, Hu et al. 2011). As a medicinal plant, ginger is widely used in traditional Chinese, Africa, Ayurvedic, and Tibb– Unani herbal medicines globally (Ali et al. 2008; Malekizadeh et al. 2012) in its various forms and during occasions to treat coughs, colds, flu, stomach upset, fatigue, combat nausea, prevent rheumatism. In the United States. Ginger serves as a remedy for motion and morning sickness, and reduce heat cramps (Semwal et al. 2015). Several studies suggest the efficacy of ginger and ginger-based products such as anti-inflammatory, antidiabetic, antioxidant (Oboh et al. 2012), anticancer (Cheng et al. 2011), including robust antimicrobial potentials against both Gram-positive (*Bacillus cereus*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) bacteria (Mahady et al. 2003, Park et al. 2008).

As seen, there is vast information available on traditional uses of *Capsicum* and ginger as well as a myriad of pharmacological activities; these characteristics increase the interest in this group of compounds. However, there is insufficient acclaimed antimicrobial activities and an identifiable mechanism to validate their potential therapeutic benefits especially against multidrug-resistant *Staphylococcus strains* and plasmid-mediated resistance in bacteria. Thus, this study is aimed at evaluating the effect of major pungent compounds from chilli pepper, and ginger for in-vitro antibacterial activities against a panel of clinically relevant MRSA stains and some Gram-negative bacteria, and their inhibitory activity against R-plasmids harboured in *E. coli*.

## **Material and Methods**

### **Plant material, extraction and compounds**



Dried powdered *Capsicum annum* L. fruit was purchased from Herbs in the Bottle Company, UK. The authentication of the dried powdered sample was done using TLC and compared against capsaicin standard. The voucher specimen (No. Cap-001) was deposited at the herbarium in the UCL School of Pharmacy. The powder (500 g) exhaustively extracted by cold agitation with 1.5 L methanol in an ultrasonic bath for 24 h. The reddish extract (3.5 g) was dried under vacuum on a rotary evaporator.

Reagents 6-gingerol (Batch number 52667, PubChem CID: 442793), 6-shogaol (Batch number 01524, PubChem CID: 5281794), nonivamide (Batch number 15970, PubChem CID: 2998), capsaicin  $\geq 95\%$  (HPLC) (Batch number M2028, PubChem CID: 2548). Other reagents from Sigma were of  $\geq 98\%$  (HPLC) purity and TLC testing done to confirm the presence of the compound. All reagents and solvents were purchased from Sigma-Aldrich Chemicals Company UK and water purified in a Millipore Milli-Q system (Merck, Bedford MA).

### **Chromatographic and NMR structure elucidation**

$^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectra obtained from a Bruker AVANCE CP QNP 500 MHz instrument (Bruker UK Ltd., UK) for elucidating the structures of the pure compounds. Chromatographic separations and isolation of capsaicin and dihydrocapsaicin included thin layer chromatography (TLC), solid phase extraction (SPE) on silica gel GF254 (0.25 mm; Merck, UK) and high-performance liquid chromatography (HPLC) coupled with the diode-array detector (DAD) (Agilent, UK). The mass spectrum was recorded with a matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE Pro; Applied Biosystems, UK) at the UCL School of Pharmacy (London, UK).

### **Bioassay-guided fractionation and isolation of active compounds**

At a first step in the isolation of antimicrobial and antiplasmid compounds, 700mg of the crude methanol extract of *C. annuum* was fractionated using solid phase extraction (SPE). A step gradient from 100% distilled water to 100% methanol was applied as the eluent system, followed by a step gradient up to 100% ethyl acetate to wash off residual material from the column. Small portions of all fractions (CA 1- 12) obtained were subjected to TLC analysis and spots on TLC were visualised by long (365 nm) and short (254 nm) wavelengths as well as being sprayed with 1% (w/v) vanillin-sulphuric acid and heated until a colouration was observed. Using TLC profiling, similar fractions were monitored and combined appropriately. Using a reverse phase TLC solvent mixtures of methanol-water (8:2) yielded Ca-A from fractions (Fr) 1 &2 and Ca-B from Frs 3-7 while Fr 8 & 9 combined to give Ca-C shown similar spots using a solvent mixture of methanol-water (9:1) with drops of acetic acid. Frs 10 & 11 showed spots on the TLC and considered as independent samples. The combined fractions Ca-A, B and C and respective fractions 10, 11, 12 were all evaluated for anti-conjugal transfer of model plasmids TP114, PKM 101 and PUB 307 using the broth mating assay. Capsaicin, obtained from Sigma-Aldrich UK was used as positive control. Bioactive Frs 10 and 11 showed an overlapped single spot on TLC plate and submitted for further NMR analysis that revealed the signals of Fr. 10 as a pure compound, 1. The <sup>1</sup>H NMR spectrum of Fr. 11 showed the major signals of capsaicin in a mixture of other compounds leading to further HPLC analysis.

### **High-Performance Liquid Chromatography analysis of sub-fraction 11**

Sub fraction 11 was characterised using HPLC-DAD according to the method described by Ng and Reuter 2005, PerkinElmer, USA with modification. The chromatographic separation was done using Agilent 1100 pump series coupled with a diode array detector (Agilent, Palo Alto, CA, USA), including an RP Nova-Pack C18 column (300 × 3.9 mm) (Phenomenex, UK) packed with 4 µm particles and a pre-column containing the same packing material, all

instrument was available in Simon Gibbons lab. All diluents and solvents used were HPLC grade and filtered using 0.22µm filters, and Fr 11 dissolved with 500µL methanol. The mobile phase solvent system comprised methanol-water from A-B 30% - 70% to A-B 70% - 30% for 20 minutes, and 10-µL aliquots injection volume at a flow rate of 1 mL/min at wavelength 254 nm and 300C. With gradient elution, the standard solution contained a mixture of capsaicin and dihydrocapsaicin while the test sample Fr. 11 and Fr-10 were included for further validation. These compounds were identified by comparison of their retention times and spectra of each peak compared to literature.

### Microbial strains and plasmids

Prof Simon Gibbons provided the bacteria strains used in the study, and Dr Paul Stapleton contributed the plasmid strains. *Staphylococcus aureus* strains include: SA1199B (norfloxacin-resistant, mediated by drug efflux), *S. aureus* SA13373 (methicillin-resistant), MRSA 12981 (methicillin-resistant), MRSA 274829 (methicillin-resistant), MRSA 774812 (methicillin-resistant), MRSA 346724 (methicillin-resistant), ATCC 25923 (standard susceptible *S. aureus* strain), EMRSA-15 and EMRSA-16 (UK epidemic MRSA strains), XU212 (MRSA and TetK-producer), *S. aureus* RN4220 (macrolide-resistant), *Enterococcus faecalis* 13379 (vancomycin-resistant), *E. faecalis* 12697 (vancomycin-resistant), *E. coli* NCTC 10418, *P. aeruginosa* 10662, *K. pneumoniae* 342, *Bacillus subtilis* BS01 and *Proteus sp* P10830. The Plasmids used with their characteristic host and resistance markers were represented in Table 1. The bacterial strains and plasmids cultured on nutrient agar slopes and incubated for 24 h at 37 °C before MIC and broth mating assay. An inoculum turbidity equivalent to a 0.5 McFarland standard ( $1 \times 10^8$  CFU/mL) was prepared in normal saline for each test organism and was then diluted 1:100 in Mueller-Hinton broth just before inoculation of the plates.

### **The minimum inhibitory concentration determination**

A volume of 100 $\mu$ L of sterile Mueller-Hinton broth (Oxoid, Basingstoke, UK) containing 20 mg/L of Ca<sup>2+</sup> and 10 mg/L of Mg<sup>2+</sup> was dispensed into the wells of a 96-well microtitre plate (Nunc; 0.3 mL total volume per well). All antibacterial agents were dissolved in dimethyl sulphoxide (DMSO) and were diluted in Mueller-Hinton broth to give a stock solution. Then, 100 $\mu$ L of the antibacterial agent stock solution (2 mg/L) was serially diluted into each well and 100 $\mu$ L of the bacterial inoculum was added to each well to give a final concentration range of 1–512 mg/L. All procedures were performed in duplicate, and the plates were incubated for 18 h at 37°C. Then, 20  $\mu$ L of a 5 mg/L methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich Ltd, Gillingham, UK) was added to each well and was incubated for 30 min. Blue colouration indicated bacterial growth. The MIC was recorded as the lowest concentration at which no colour change observed.

### **Plasmid inhibition by crude fractions and compounds**

The sub-inhibitory concentration (SIC) of 100 mg/L used throughout the assay was 0.25  $\times$  MIC of the test samples against *E. coli* NCTC 10481 (data not shown). Plumbagin at a concentration of 8 mg/L was used as a positive control. The test samples Ca-A, Ca-B, Ca-C and Frs 10-12, capsaicin, dihydrocapsaicin, nonivamide, 6-gingerols and 6-shogaol were all evaluated for their ability to inhibit the conjugal transfer of model plasmids TP114, PKM 101 and PUB 307 using broth mating method described by Rice and Bonomo 2005 with some modifications. Mating between the plasmid-containing donor strain *E. coli* K12 J53 and the recipient *E. coli* ER1793 was performed in Luria-Bertani broth. Transconjugants were identified by plating bacterial mixtures onto selective media containing the appropriate

antibiotics: streptomycin (to select for the recipient) plus either ampicillin (to detect the transfer of pKM101 and pUB307) or kanamycin (TP114). Ampicillin and nalidixic acid were used to identify the transmission of R7K or R6K to the recipient *E. coli* JM109. The concentration of the various antibiotics used was 30 mg/L.

The plasmid transfer inhibition frequency was expressed as the number of transconjugant colonies (CFU/mL) per recipient (CFU/mL) and was represented as a percentage of transfer exhibited by a plasmid in the absence of the test compound (normalised to 100%): %

Inhibition Transfer frequency CFU/mL =

$$\frac{\text{Number of transconjugants} \times 100}{\text{Total number of donor carrying cells}}$$

Total number of donor carrying cells

The rate of inhibition means that the transfer frequency of the plasmid is inversely proportional to inhibition of the drug such that the lower the transfer frequency of the plasmid, the higher the inhibition by the drug, and vice versa. It is categorised into three levels of inhibition relative to the positive control: active if transfer frequency value falls between the range 0-10 %, moderate, when the values are within 15 - 50%; and poor or no activity when the values are 50% and above. In some cases, no inhibition may be observed, and the transfer frequency value may become higher than the control, suggesting enhancement of the plasmid transfer by antagonist mechanism. The results based on at least three independent experiments. Data were expressed as mean $\pm$ SD. Differences between the two mean values were calculated by Student's t-test. P-values of <0.05 were considered statistically significant and P < 0.01 as very significant.

## Results

### **Chromatographic isolation and elucidation of capsaicinoids**

Capsaicin (1) and dihydrocapsaicin (DHC) (2) (Fig 1b) were responsible for the inhibition of bacterial growth or the transfer of plasmids; pKM101, PUB 307, TP114 in *E. coli*. The elucidation of the structures was done using detailed one-dimensional and two-dimensional NMR experiments, mass spectrometry and concerning existing NMR data in the literature. The NMR assignments of the compounds are shown in Table 2. The chemical structure resemblances of 6-gingerol (3), 6-shogaol (4) and nonivamide (5) are also shown in Fig 1c. The chromatographic fingerprint (Fig 2) showed separation of capsaicin and dihydrocapsaicin at 6.26 min and 12.60 min intervals at 280 nm wavelength.

### **The in vitro antibacterial activity of the compounds against multidrug-resistant *S. aureus* and Gram-negative bacteria**

The antibacterial activities of capsaicin, dihydrocapsaicin (DHC), nonivamide, 6-gingerol, and 6-shogaol were assessed against a panel of multi-drug resistant Gram-positive and Gram-negative bacteria shown in Table 3. Capsaicin had a poor MIC value of 256 mg/L on the test bacteria except for *B. subtilis* (MIC =128 mg/L). DHC moderately inhibited the growth of *B. subtilis* and *E. faecalis* only with MIC value of 128 mg/L. Nonivamide was active against XU 212 at a recommendable MIC of 64 mg/L, followed by RN4220, MRSA 12981, EMRSA-15 and *E. faecalis* 13327 at MIC of 128 mg/L over poor activity against other test organisms (MIC=256-512 mg/L). The most potent activity was by 6-gingerol and 6-shogaol (MIC=8 mg/L) against pathogenic *E. faecalis* and RN4220 that is four-fold lower than erythromycin at MIC value of 32 mg/L. Generally, 6-gingerol and 6-shogaol were moderately active against these problematic multidrug-resistant strains within a remarkable MIC range of 8-12 mg/L, Noteworthy, is the susceptibility of NorA-expressing SA1199B and XU212 strain expressing TetK efflux mechanism at MIC of 16 mg/L to gingerol. All the compounds had

weak activity against the Gram-negative bacteria at MIC of 512 mg/L except 6-shogaol with an active MIC value of 128 mg/L against *E.coli* NCTC 10418, *P. aeruginosa* 10662, and *K. pneumoniae* 342.

### **The antiplasmid activity of fractionated extracts, isolated capsaicinoids and capsaicin analogues on the inhibition of R-plasmid conjugal transfer**

The crude methanol extract and sub-fractions of *Capsicum annum* were evaluated for their effects on plasmid inhibition assay. The samples demonstrated various rates of inhibition of the three plasmids tested compared to the control (Figure 3). Interestingly, sub-fraction Ca-SPE 11 stood out as the most active, among all fractions, against the transfer of plasmids PKM 101, PUB 307, TP114, followed by the crude extract of *C. annum* and capsaicin independently. These results are indicative that the crude extract and semi-fractionated drugs contained active compounds leading to their practical pharmacological effect more than the single compounds typified in capsaicin. Usually, crude extracts act in synergism with other components of the extract that may or have no pharmacological activity. Many of such cases are known, and such crude drugs provide a good basis for chemical quality control and quality assurance of the synergistic and pharmacological roles that give credence to their use in traditional herbal medicines. In Figure 4, capsaicin showed reproducible rates of selective but active inhibition of resistant plasmids R7K, PUB307 and PKM 101 at transfer frequencies of 5.03%, 9.78% and 13.05% respectively. However, the transfer of TP114 and R6K were enhanced, hence the antagonism of plasmid inhibition. The rates of transfer of PUB307 were affected mostly by the presence of dihydrocapsaicin (3.34%), 6-gingerol (1.22%), and 6-shogaol (2.90%). The rates of transfer inhibition of TP114 were equally active within the range of 0.14 %-7.2 0% (Figure 5). In the case of PKM 101, the effect of 6-gingerol and 6-shogaol reduced the rate of transfer of the plasmid, but inhibition was moderate by DHC and nonivamide. Nonivamide recorded its highest inhibitory activity against TP114 at 7.19% and

PUB 307 at 22.16%. DHC had a promising significant effect against PUB 307 at 3.33%. There was no inhibition effect of DHC against TP114; instead, an antagonist effect was observed.

## Discussion

Plants and herbs contain different classes of structurally similar phytochemicals, and their antimicrobial abilities can be promising. Growing interest in capsaicinoids, particularly capsaicin and dihydrocapsaicin has led to its characterisation widely with high-performance liquid chromatography method (Peng et al. 2009). Researchers have recommended this method for rapid isolation and easy detection of capsaicinoids due to the similarity in their structures as was the case with the separation of dihydrocapsaicin from capsaicin in this study. To the best of our knowledge, there are no previous studies on the activity *Capsicum* extracts or any of the capsaicinoids and gingerol focusing on harnessing their antiplasmid and antibacterial effect on a wide range of MRSA.

Following the results, the antimicrobial activity of capsaicin and dihydrocapsaicin for almost all Gram-positive MRSA and Gram-negative organisms tested revealed low MIC values from 512-256 mg/L since natural products are considered as weak or potent inhibitors of microbial activity when MIC values are higher than 100 mg/L or lower than 25 mg/L respectively (Cos et al. 2006). Santos et al. 2015 earlier reported that capsaicin and DHC strongly inhibited *Streptococcus mutans* at remarkable MIC of 1.25 mg/L, including studies from Koffi-Nevry, 2011, Careaga et al. 2003 and Mahady et al. 2003, which showed that capsicum extract or capsaicin is responsible for microbial activity against wide range of organisms. These reports are in contrast to poor MIC values of 256-512 Mg/L recorded in our study indicative of no inhibition of growth against *Enterrococcus*, *Klebsiella*, *E. coli*, *Pseudomonas*, *Proteus* and MRSAs. A similar level of microbial inactivity was observed in DHC, except moderate



activity against *E. faecalis* and ATCC 29523 at MIC 128mg/L, and capsaicin against *B. subtilis* BsSOP01 respectively. On the surface, it is no surprise that the MRSA and Gram-negative organisms are resistant. MRSA are known to possess efflux pumps such as SA1199B (NorA), RN4220 (MsrA), and XU212 overexpressing TetK (tetracycline) efflux pump that reduces the transport and recognition of tetracycline (Gibbons, 2008), thereby rendering the drugs clinically inactive while the impermeability of Gram-negative bacterial membrane is also considered as resistance mechanism. Perhaps, the increased inhibitory activity of capsaicin and DHC can be achieved in the combination of an antibiotic, as was the example of ciprofloxacin and capsaicin to suppress the presence of efflux pump NorA responsible for resistance of *S. aureus* to Norfloxacin (Kalia et al. 2012).

Nonivamide displayed a moderate activity against *Bacillus subtilis* BsSOP01 and *E. faecalis*, including EMRSA-15 and MRSA 12981 strains at MIC value of 128 mg/L. Interestingly nonivamide, the synthetic analogue of capsaicin acts similarly as an agonist of VR1 vanilloid/TRPV1 receptor produced similar antibacterial activity overall but selectively increased activity against MRSA and *E. faecalis*. Reports of antimicrobial activities of nonivamide are still scanty, but the compound is considered more heat stable than capsaicin (<https://pubchem.ncbi.nlm.nih.gov/compound/N-Vanillylnonanamide>), which could be responsible for the enhanced antimicrobial activity observed. 6-shogaol and 6-gingerol showed a spectrum of antibacterial activities ranging from weak MIC of 512 mg/L against EMRSA16, MRSA 12981, and most of the Gram-negative organisms, to robust inhibition of *E. faecalis* and RN 4220 at 8mg/L. The presence of MrsA pump in RN4220 could cause the weak effect of 6-gingerol by expelling them from their drug-binding sites (Mishra et al. 2012, Ross et al. 1995). All compounds except 6-shogaol were poorly active against the Gram-negative bacteria. Perhaps, the Gram-negative microorganisms are not susceptible to the compounds due to lack of permeability barrier to the compounds, or apparently, the

compounds become modified resulting in loss of activity. The strong MIC values of 6-shogaol and 6-gingerol are in line with published literature that they effectively inhibited the growth of oral pathogens at a minimum inhibitory concentration (MIC) range of 6–30  $\mu\text{g/mL}$  (Park et al. 2008).

The structurally similar vanillyl amide moieties of these compounds in Gingerol (GN) ((5S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decan-3-one), shogaol (SG) ((E)-1-(4-hydroxy-3-methoxyphenyl) dec-4-en-3-one) and capsaicinoids (vanillyl (4-hydroxy-3-methoxyphenyl) ketone) is notable. Dihydrocapsaicin (8-methyl-N-vanillynonenamide/-N-(4-hydroxy-3-methoxybenzyl)-8-methylnonanamide), is a reduced 6, 7 dihydro-derivative of capsaicin as a result of the degree of unsaturation of the 9-carbon fatty acid side (Garces-Claver et al. 2007). Although the chemical structures of these compounds are similar, it follows that chemically identical compounds may have significantly different biological activities depending on the binding sites properties of the substrates. In the case of capsaicin and DHC, the amount and the composition of the bioactive substances may differ according to the extraction methods, the geographic and the growing conditions, or time of the harvest (González-Zamora et al. 2013). Importantly, the chemical structure of these compounds could be modified through structure-activity studies for derivatives that may have a broader spectrum of inhibitory activity whether as a stand-alone drug or in combinatory action with other antibiotics.

The effect of capsaicin on these plasmids remain remarkable such that it exhibited a broad range of activity over unrelated plasmid incompatibility groups; Inc N, W and P, while DHC seems plasmid specific. The antiplasmid activity of dihydrocapsaicin was only notable against the conjugal transfer of amoxicillin-resistance-conferring plasmid PUB307. These results suggest the ability of the test compounds to block the plasmid conjugation processes. Not only are these broad host plasmid conduits of various antibiotic resistance determinants

in *E. coli*, they code similar pattern of conjugal replication and type IV secretion transfer system (Schroder and Lanka 2005). Plasmid TP114 confers kanamycin- (aminoglycoside) resistance (Kmr) while PKM 101, R6K and R7K confer resistance genes to many antimicrobial classes especially genes encoding amoxicillin ( $\beta$ -lactam) resistance (Amr), which illustrates the very diverse nature of plasmids (Oyedemi et al. 2016). The common inhibitory activity of capsaicin over DHC might be presumably related to the difference in saturation of the alkyl side chain. The strong inhibition effect of nonivamide on TP114 was somewhat surprising being that capsaicin and DHC only showed a minimal effect. Nonivamide is a less pungent capsaicin analogue; however, research has shown its significant results similar to capsaicin in regulating cellular responses (Rohm et al. 2013). The effect of capsaicin and dihydrocapsaicin on PKM 101 was consistent, which shows that PKM 101 was particularly sensitive to the capsaicinoids. The impact of 6-gingerol and 6-shogaol were very profound and steady against the transfer of the plasmids PKM101, TP114 and PUB307 suggesting their interference with the plasmid DNA transfer and replication (Dtr) process or the secretion proteins substrates actively involved in the bacterial conjugation (Schroder and Lanka 2005)

Both capsaicin and gingerol are agonists of transient receptor potential vallinoid 1 (TRPV 1), a  $\text{Ca}^{2+}$  permeable ion receptor in human (Geng et al. 2016) and have been shown to possess some ability to alter expression of several genes (Clark and Lee, 2016). However, it is unclear whether this molecular signalling pathway is responsible for its acclaimed antimicrobial effect. The ability of triple bond group between C-12 and C-13 of unsaturated fatty acids to inhibit conjugal plasmid transfer by targeting Dtr plasmid systems has been studied in Inc W plasmid group (Fernandez-Lopez et al. 2005). Hence, the length of the aliphatic side chain in capsaicin and 6-gingerol is optimum to exert biological activity while the decrease in the side chain alters or even lead to loss of such activity. The presence of the carboxylic group, chain

length and presence of the double bond were considered responsible for their potent inhibitory activity, and such are the characteristic features of capsaicin, DHC, 6-gingerol and 6-shogaol accountable for the observed antiplasmid activity. The marked effect of 6-gingerol and 6-shogaol on the plasmid coded type IV secretion systems (T4SS) could further be supported by earlier reported mechanistic result of gingerols, on the inhibition of *Helicobacter pylori* CagA+ and associated secretion proteins which show homologies to genes encoding T4SS components Mahady et al. 2003; Haniadka et al. 2013)

### **Conclusion**

These findings validate the biological effects and extensive use of capsicum and ginger-based preparations used in herbal medicines making them good candidates for antimicrobial drug development. Both capsaicinoids and gingerols showed a pharmacologically active effect on the plasmid conjugal transfer property; more importantly, their ability to reverse horizontal antibiotic resistance spread in bacteria although they are greatly considered as nutritional factors. These compounds are promising with a highlight on their chemical nature that can be modified to enhance their pharmacological and therapeutic benefits into deriving a new class of antimicrobial drug leads with a novel mechanism. These findings also lay the foundation for further research of these compounds and their effects on efflux pumps and plasmid resistance mechanism in bacteria.

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### **Competing interests**

The authors declared no conflict of interest

**Ethical approval**

Not required.

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Table 1: Plasmid strains used, host and resistance markers

Plasmid	Molecular weight	Incompatibility group	Host	Resistance marker <sup>(r)</sup>
PKM 101	35.4kb	IncN	<i>E. coli</i> WP2 uvrA	Ap <sup>r</sup>
PUB 307:RP1	56.4Kb	IncP	<i>E. coli</i> K12 J53	Ap <sup>r</sup> , Km <sup>r</sup> , Tet <sup>r</sup>
R6K	39.4Kb	IncX	<i>E. coli</i> K12 J53	Ap <sup>r</sup> , Sm <sup>r</sup>
R7K	30.3 Kb	IncW	<i>E. coli</i> K12 J53-2	Ap <sup>r</sup> , Sm <sup>r</sup> , Sp <sup>r</sup>
R1-drd-19	93.9 Kb	IncF11	<i>E. coli</i> K12 J53	Ap <sup>r</sup> , Cm <sup>r</sup> , Km <sup>r</sup> , Sm <sup>r</sup> , Sp <sup>r</sup> , Su <sup>r</sup>
<i>E. coli</i> ER1793	Recipient			Sm <sup>r</sup>
JM 109	Recipient			Nal <sup>r</sup>
TP114	62.1Kb	IncI2	<i>E. coli</i> K12 J53	Kmr

Km<sup>r</sup> = kanamycin, Ap<sup>r</sup> = ampicillin, Tet<sup>r</sup> = tetracycline, Sm<sup>r</sup> = streptomycin, Sp<sup>r</sup> = spectinomycin, Cm<sup>r</sup> = chloramphenicol, Su<sup>r</sup> = sulphonamide, Nal<sup>r</sup> = nalidixic acid

Table 2: NMR of capsaicin BM-8 and dihydrocapsaicin BM-9 in CD<sub>3</sub>OD (<sup>1</sup>H 500MHz, <sup>13</sup>C 125MHz)

	Capsaicin				Dihydrocapsaicin		
	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C (100MHz) in CDCl <sub>3</sub>	<sup>1</sup> H (400MHz) Kobata <i>et al.</i> , 1998	<sup>1</sup> H	<sup>1</sup> H (100MHz) in CDCl <sub>3</sub>	<sup>13</sup> C (400MHz) Kobata <i>et al.</i> , 2009
1	173.1		172.9				173.1
2	37.0	2.14, t, <i>J</i> =7.5, 2H	36.7	2.19	2.33	2.19	36.9
3	27.1	1.69, s, 1H	25.3	1.65	1.54	1.64	25.9
4	28.4	1.31, <i>J</i> =8, 2H	29.3	1.38	1.29	1.31	29.6
5	33.3	1.94, 2H,	32.5	1.98	1.29	1.29	27.2
6	127.9	5.26-5.28, m, 2H	126.5	5.30	1.29	38.9	126.0
7	139.1	5.26-5.28, m	138.1	5.37	1.25		
8	30.1	2.15, t, <i>J</i> =7.5 2H	31.0	2.20	1.62	1.50	28.0
9, 10	23.1	0.70-0.88, dd, <i>J</i> =7.5 6H	22.7	0.95	0.91	0.86	22.7
1'	131.6		130.3				130.3
2'	112.5	6.78, s, 1H	110.7	6.79	6.85	6.84	114.5
3'	149.0		146.8				145.2
4'	146.8		145.2				146.8
5'	116.1	6.65, d, <i>J</i> =8.5, 2H	114.4	6.74	6.71	6.79	110.8
6'	121.4	6.65, d, <i>J</i> =8.5, 2H	120.7	6.80			120.8
7'	43.9	4.20	43.5	4.33	4.14	4.33	43.6
OCH <sub>3</sub>	56.4	3.76, s, 3H	55.9	3.85	3.84	55.9	3.85
OH		5.16		5.87	5.35		5.89
NH		5.20		5.84	4.59		

Table 3: Minimum inhibitory concentrations (MICs) of the agents against various Gram-positive and Gram-negative bacteria.

Strains	Minimum inhibitory concentration (mg/L)									
	Cap	DHC	Noni	6-gin	6-sho	Nor	Tet	Ery	Oxa	Cip
SA1199B	256	512	256	16	16	32				
ATCC 29523	256	128	256	128	128	32				
<i>Bacillus subtilis</i> BsSOP01	128	256	128	64	NT	0.25				
XU212	256	256	64	16	16	0.25				
EMRSA 15	256	512	128	64	32		16			
EMRSA 16	256	512	512	512	512		0.25			
RN 4220	256	512	128	128	8		0.25			
MRSA 346724	256	512	256	128	16			32		
MRSA 774812	256	512	512	512	512				<0.25	
MRSA 274829	256	256	512	64	64				<0.25	
MRSA 12981	256	512	128	512	128				128	
<i>Enterococcus faecalis</i> 13379	256	128	128	8	NT				8	
<i>E. coli</i> NCTC 10418	512	512	512	512	128					<0.06
<i>Pseudomonas aeruginosa</i> 10662	512	512	512	512	128					≤0.06
<i>Klebsiella pneumoniae</i> 342	512	512	512	512	128					≤0.03
<i>Proteus sp</i> P10830	512	512	512	512	512					≤0.03

Cap= capsaicin, DHC= dihydrocapsaicin, Noni= nonivamide, 6-gin= gingerol, 6- sho= shogaol, NT= Not Tested

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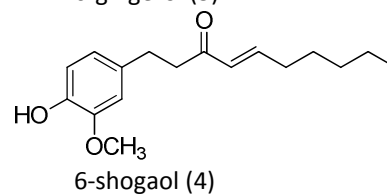
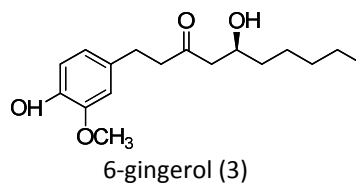
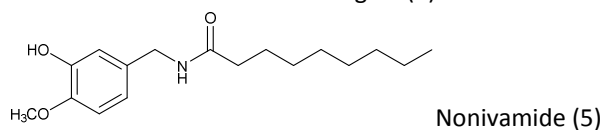
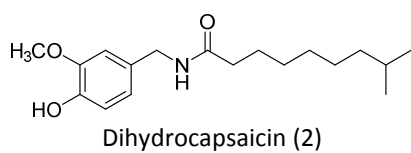
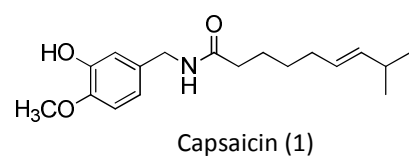
(a) *Capsicum annum*(b) *Zingiber officinale*

Figure 1. (a) Diagram of *Capsicum annum* L. (b) *Zingiber officinale* Roscoe, (c 1-5) chemical structures showing structural resemblances with capsaicin, dihydrocapsaicin, nonivamide, 6-gingerol and 6-shogaol.

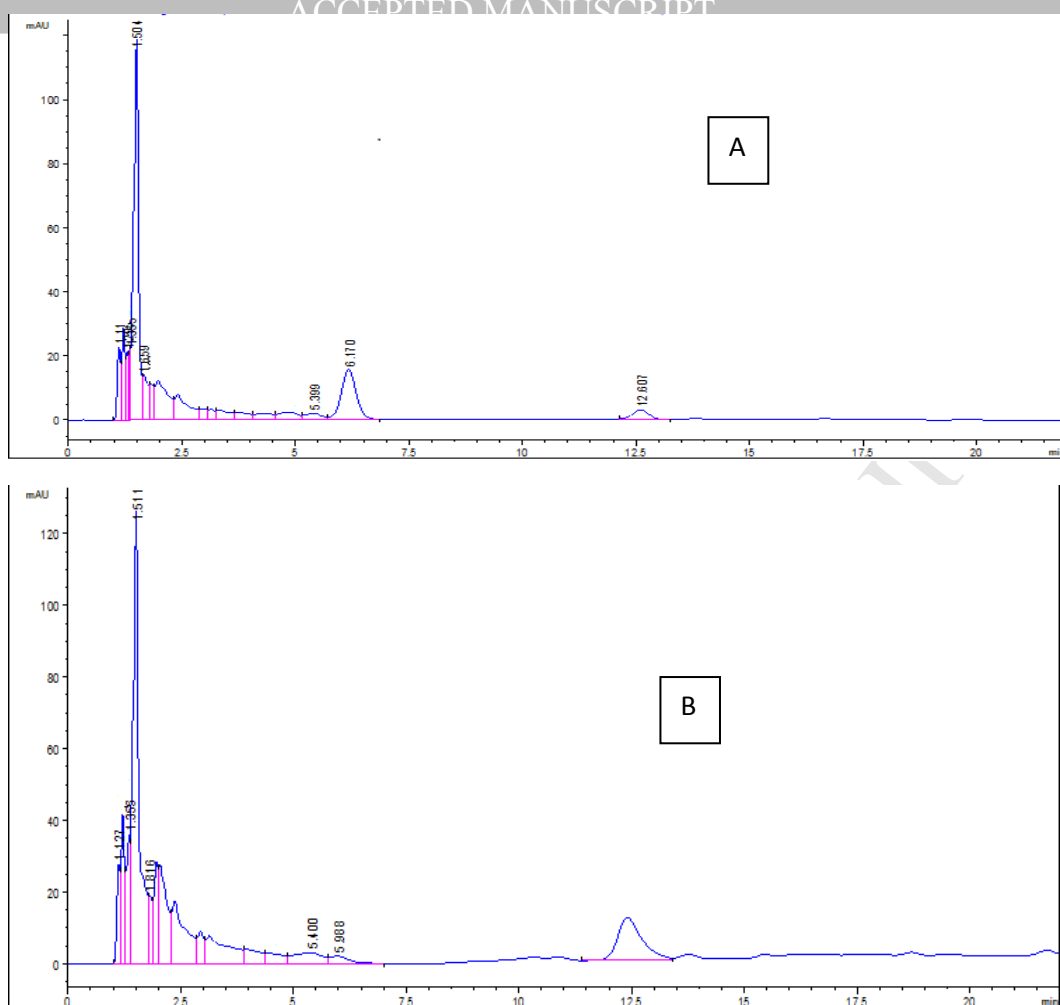


Figure 2: HPLC-DAD chromatograms of capsaicin (A,  $t_1=6.17$  min) and dihydrocapsaicin (B,  $t_2=12.61$  min) according to their corresponding peaks at 254nm.

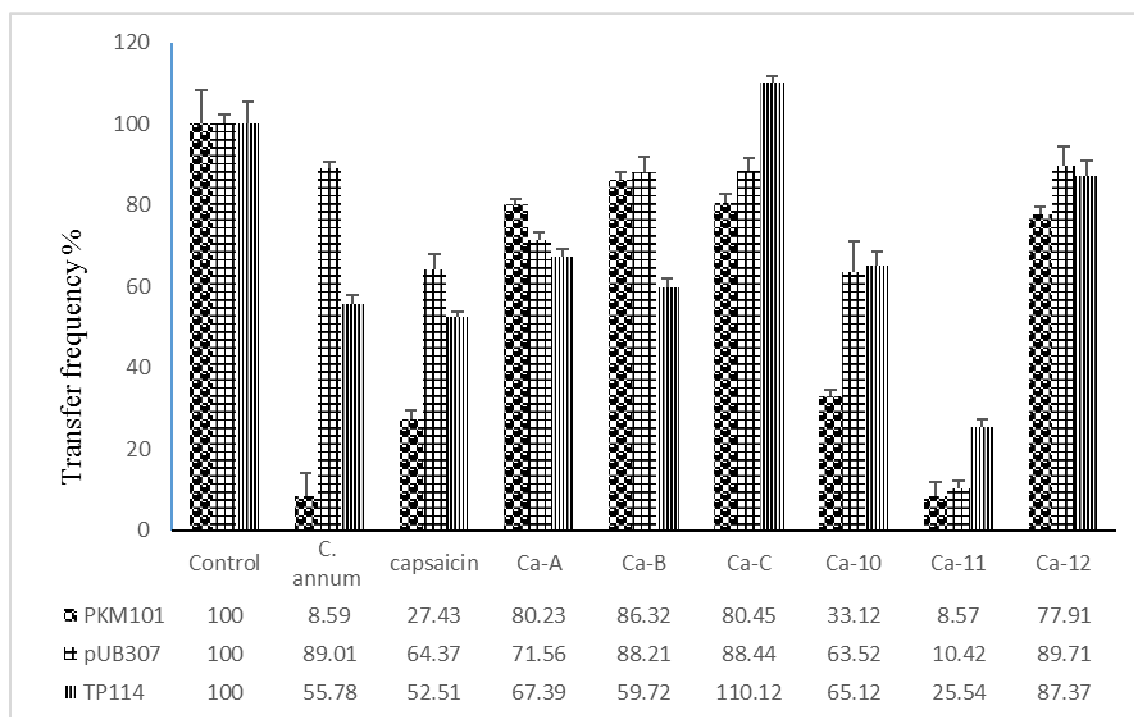


Figure 3. Plasmid transfer inhibition in the presence of crude *C. annum* extract, SPE fractionated extracts Ca-A, -B, -C, -10, -11, -12 and capsaicin (100mg/L). Plasmid transfer in the absence of the drugs is normalised to 100%. Results are expressed as the mean  $\pm$  S.D. (n = 3). \*P < 0.05 and \*\*P < 0.01 versus positive control at the same point. SIC, subinhibitory concentration.

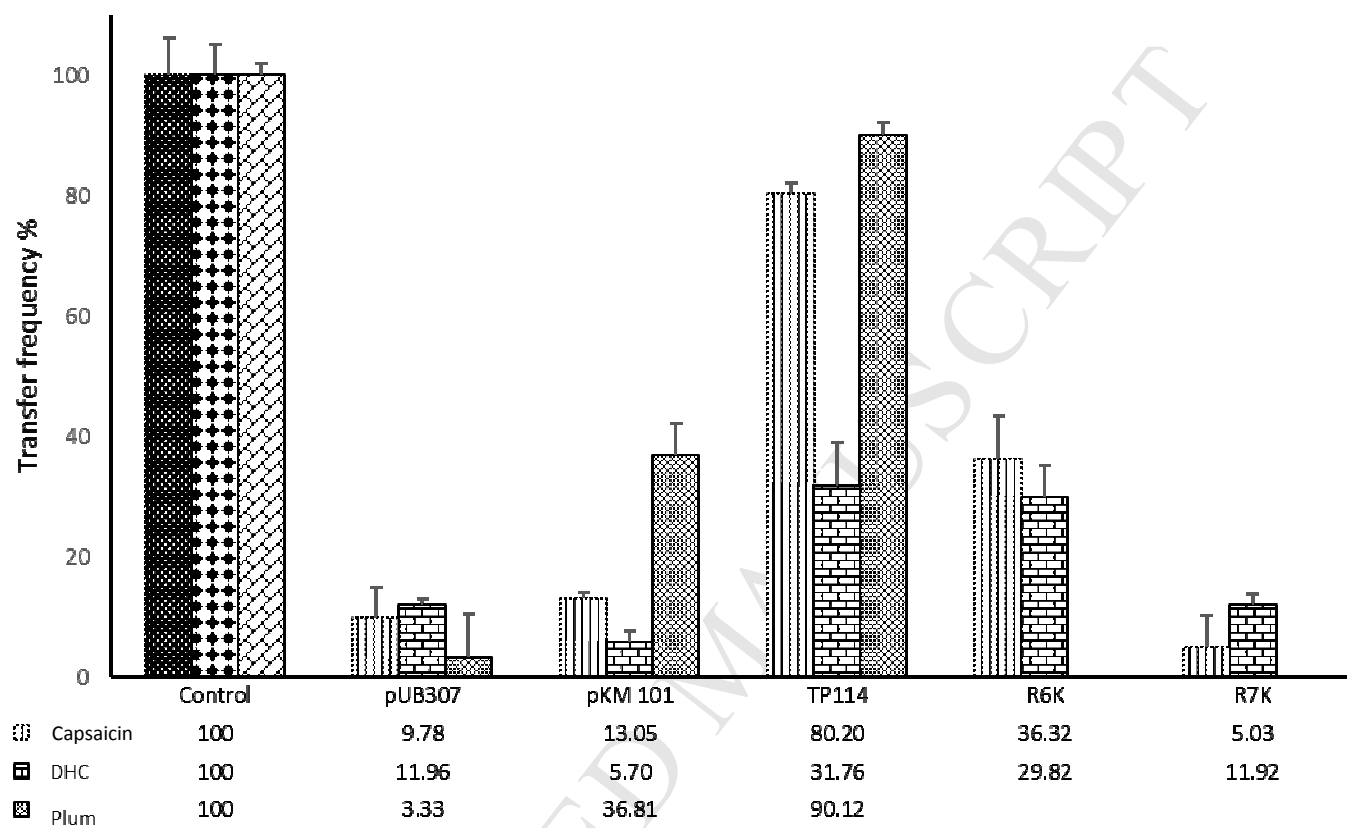


Figure 4. Plasmid transfer inhibition in the presence of capsaicin, dihydrocapsaicin (100mg/L) and plumbagin (SIC 8 mg/L). Plasmid transfer in the absence of the drugs is normalised to 100%. Results are expressed as the mean  $\pm$  S.D. (n = 3). \*P < 0.05 and \*\*P < 0.01 versus positive control at the same point. SIC, subinhibitory concentration



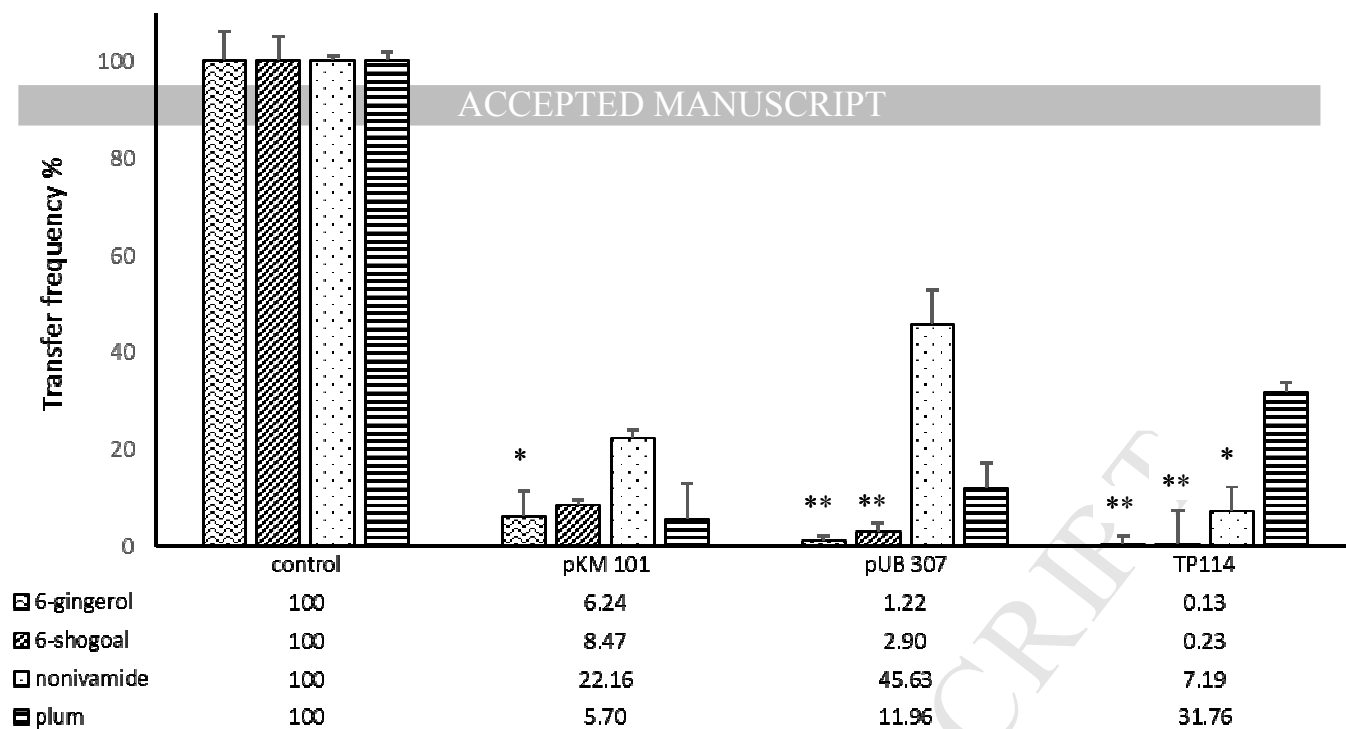


Figure 5. Plasmid transfer inhibition in the presence of 6-gingerol, 6-shogaol, nonivamide (100mg/L) and plumbagin (SIC 8 mg/L). Plasmid transfer in the absence of the drugs is normalised to 100%. Results are expressed as the mean  $\pm$  S.D. (n = 3). \*P < 0.05 and \*\*P < 0.01 versus positive control at the same point. SIC, subinhibitory concentration

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