Antibacterial and hemolytic efficacy of olive oil based *Tridax procumbens* herbal cream

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Abstract

Herbal based creams are being increasingly researched as an alternative to synthetic ointments and creams. In the present study, the herbal cream prepared using methanolic extract of Tridax procumbens and olive oil was tested for the antibacterial on clinical bacterial isolates and hemolytic activity on human RBCs. The GC-MS analysis of methanolic extract of Tridax procumbens shows the major compounds Phenol, 2, 4-bis (1,1-dimethylethyl)-, phosphite (3:1) (37.71%), Methyl commate D (29.06 %), Methyl commate A (13.25%), Stigmasterol (6.1 %) and Gamma.-sitosterol (3.37 %) which posses antimicrobial, antioxidant, anti-inflammatory and antibiofilm activities. Whereas the GC-MS results of olive oil shows 2 compounds Squalene (98.958 %) and Farnesol isomer A (1.042 %) which exhibit antibacterial and wound healing efficiency.

The antibacterial activity tested using agar well diffusion and antibiofilm assay against 4 clinical pathogens namely E. coli, S. aureus, P. aeruginosa and K. pneumoniae shows that herbal cream has higher microbial reduction capacity on four clinical pathogens. Further the herbal cream exhibits minimal hemolytic action 0.06 % on human erythrocyte at 100 µg/mL. Hence the prepared herbal based cream can be used for traumatic wounds in clinical ambience for wound healing.

Keywords: *Tridax procumbens*, Olive oil, antibacterial activity, antibiofilm activity, hemolysis.

Introduction

Herbal based products plays a vital role in medical sector due to their low side effects, better and faster recovery in many treatments and therapies. Herbal preparation like emulsion, cream and ointment are applied topically for treatment of wounds stimulating the healing process with their bioactive compounds[1]. Most plants have antioxidant, antimicrobial and anti-inflammatory property that helps in short term tissue regeneration.

Some of commercially available products produce scars, hyper pigmentation and long term recovery in topical wounds[2]. To avoid such traits and to minimize the side effects, herbs and their phytoconstituents are used in wound healing. The herb *Tridax procumbens* (family: Asteraceae) commonly known as 'coat button' is used in regular traditional practice to cure dermal lesions[3].

Earlier reports have identified that the herb *T. procumbens* has antimicrobial[4], antioxidant and anti-inflammatory properties[5] and bioactive compounds of that herb enhance wound recovery in short period of time[6]. Formulation of antibacterial creams use hemolytic assay as a gold standard for testing the toxicity of product[7].

To minimize the hemolytic and cytotoxicty of the prepared product, olive oil is mixed in cream preparation[8]. Olive oil also possesses promising antibacterial activity against pathogens[9] and promotes collagen deposition and stimulating Nrf2 expression on wound[10]. This study focuses on the formulation of *T. procumbens* methanolic extract *and* olive oil as an antibacterial herbal cream, olive oil alone and methanolic extract of *T.procumbens* were compared for their efficacy against clinical pathogens and human erythrocyte.

Material and Methods

Materials required: Extra virgin olive oil, Sodium dodecyl sulphate (SDS), Muller Hinton Agar, Phosphate buffered saline pH 7.4, Trypic soy broth, 33% Acetic acid, Crystalline violet, Triton X 100 and Poly ethylene glycol 400 (PEG).

Collection and extraction of *Tridax procumbens*: *Tridax procumbens* plants were collected in and around Salem, Tamil Nadu, India (Latitude 11.7184° N and Longitude 78.0771° E). The leaves were washed in double distilled water and dried in shade for 1 week. Dried leaves were grinded as powder. The extraction procedure was performed with soxhlet apparatus. 100 g *of T. procumbens* powdered leaf was added to 500mL of methanol and the temperature was maintained at 64 °C for 24 hrs. The methanolic extract of plant was filtered through Whatmann no.1 filter paper. The excess solvent was dried in rotary vacuum evaporator and dried samples were stored under 4 °C in refrigerator for further use[11].

Preparation of cream: Extra virgin olive oil was purchased from local market of Salem, Tamil Nadu, India was used as oil phase for cream preparation. Cream was formulated in 1:1 ratio of aqueous phase and oil phase. 10mL of olive oil was heated at 80°C in double boiler method with 0.5 g of bees wax. 1g of dried methanolic plant extract was mixed in 10mL of double distilled water and placed at water bath at

80°C. 18 mg of Sodium dodecyl sulphate (SDS) was added to aqueous phase and allowed to dissolve completely. Aqueous phase was slowly added into oil phase with continuous stirring until its forms as homogenous cream for about 15 mins[12].

GC-MS analysis: Filtered olive oil sample 100 µL was mixed in 2 ml of DCM (dichloromethane) for GC-MS analysis and 100 µg of *Tridax procumbens* methanol extract powder was mixed with methanol for GC-MS run. Shimadzu GCMS QP 2020 fused silica column were used for analysis, packed with SH-Rxi-%Sil MS (30 m × 0.25 mm ID × 250µm df) using helium as carrier gas at a constant flow for components. The injector temperature was set at 280°C during the chromatographic run. The 1µL of samples were injected separately into the instrument the oven temperature was as follows: 40 °C (2 min) followed by 280 °C at the rate of 10 °C min^{-1} and 280 °C, where it was held for 3 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The components spectrum was compared with the database of spectrum of known components stored in the GC-MS NIST (2017) library.

Antibacterial activity: Freshly prepared LB broth was inoculated with clinical pathogens (*E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae*) at 37 °C for 24 hour. Antibacterial test was performed with a well diffusion agar method. The cultures were swabbed over the solidified Muller Hinton Agar. The bored wells in agar were impregnated with 20-100 µg/mL of *Tridax procumbens* extract, olive oil and mixture of both as herbal cream. Each plate was incubated overnight at 37 °C. Zone of inhibition was measured in mm. Double distilled water was used as control and tests were performed statistically with triplets.

Antibioflim activity: 10 µl of freshly prepared clinical isolates were inoculated in 190 µL of Trypic soy broth (TSB) in 96 well microtitre plate. 100 µl of distilled water is added to maintain the water loss in medium. The 4 bacterial isolates were inoculated with different concentration of *Tridax procumbens* leave extract, olive oil and freshly prepared cream of both combinations (20-100 µg/mL). The plates were incubated at for 24 hrs at 37 °C. After incubation, the micro titre plates were washed with run tap water about 3-4 times.

Then 200 µL of crystalline violet should be added in each well and after 15 min, rinse the excess stain with water and air dry. The biofilm cell bounded crystalline violet was dissolved by 33% of acetic acid. Then 100 µL of each destained solution was transferred to fresh microtitre plates. The reduction rates of biofilm for different concentration of 3 products were measured in terms of OD_{550nm}.

Hemolytic assay: Hemolytic assay was performed by blood of healthy donor as described briefly in our previous study[13]. 5mL of blood was drawn and used to calculate the RBC lysis for different concentration (20-100 µg/ml) of *T. procumbens* leaf extract, olive oil and prepared cream. 1% of Triton X-100 and 40% of Poly Ethylene Glycol (PEG) was used as positive and negative control respectively.

PBS diluted heparinized blood was incubated with different concentration of 3 individual products as listed above with the concentration (20-100 µg/mL) in triplicate at 37 °C for 3 hrs in water bath. After incubation the samples were centrifuged at 3000 rpm for 10 mins.

The supernatant was collected individually in microtitre plate and absorbance was measured in OD_{570nm}. The hemolysis was calculated in terms of percentage with formulae [H (%) = (OD_{570nm} sample – OD_{570nm} PEG 40%) / (OD_{570nm} Triton X-100 1% - OD_{570nm} PEG 40%) × 100**]**. The results were expressed in Mean ± SD (n=3).

Statistical analysis: All the experimental data Mean ± SD (n=3) was subjected for (One Way ANOVA) to calculated the significance of P valve between and control and treated *T.procumbens* leaf extract, olive oil and prepared cream. Statistical analyses were performed with Graphpad PRISM (Version 4.0). The Dunnett's test of One Way ANOVA indicates significance values as ($p<0.05$= '*'), ($p<0.01$= '**') and ($p<0.001$='***').

Results and Discussion

GC-MS analysis of *Tridax procumbens* extract and Olive oil: GC-MS spectrum results confirm the presence of miscellaneous compounds eluted at different retention time for both *T. procumbens* and olive oil respectively (Figure 1 and 2). GC-MS analysis of *T. procumbens* extract leads to identification of 15 components during GC run. The compounds present in entire herb *T. procumbens* were detected by GC-MS and their biological significance was listed in table 1.

Phenol, 2, 4-bis(1,1-dimethylethyl)-, phosphite (3:1) (37.71%), Methyl commate D (29.06 %), Methyl commate A (13.25%), Stigmasterol (6.1 %), Stigmasterone (4.23%), Gamma.-sitosterol (3.37 %), Neophytadiene (1.99%), Decane, 1-iodo-(1.76%) and Benzene, (1methyldodecyl)-(1.27%) were the 10 major compounds, N-Hexadecanoic acid (0.96%), 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-(0.92%), 2,6,10-trimethyl,14-ethylene-14-pentadecne (0.68%), 6.beta.Bicyclo[4.3.0] nonane, 5.beta.-iodomethyl-1.beta.-isopropenyl (0.69%) and Undecane (0.46%) were 5 minor compounds present in methanolic extract of *T.procumbens.*

GC-MS analysis of Olive oil showed the presence of 2 main components namely Squalene (98.958 %) and Farnesol isomer A (1.042 %) were indicated in table 2. These compounds have antimicrobial, antioxidant, anti-inflammatory and antibiofilm potential[29-31].

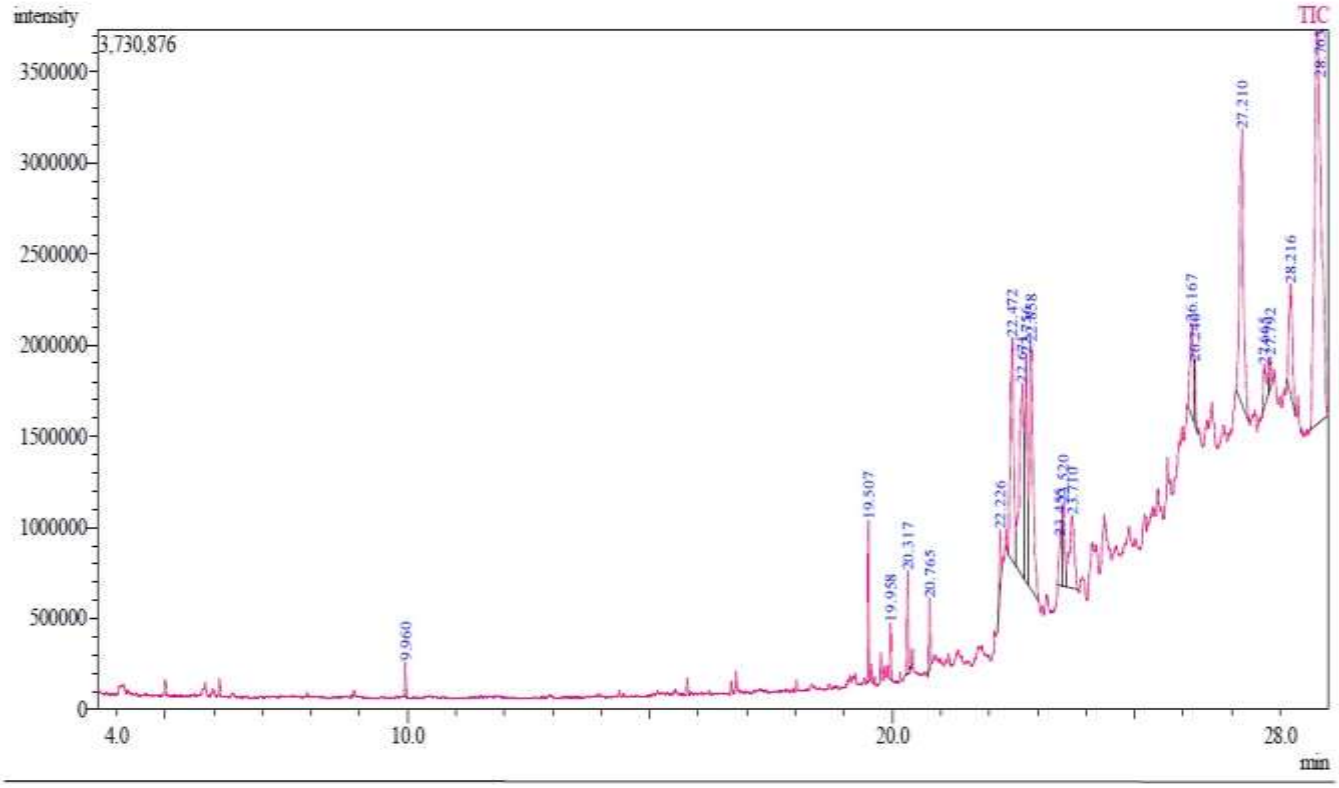

Figure 1: GC-MS chromatogram of methanolic extract of *Tridax procumbens*

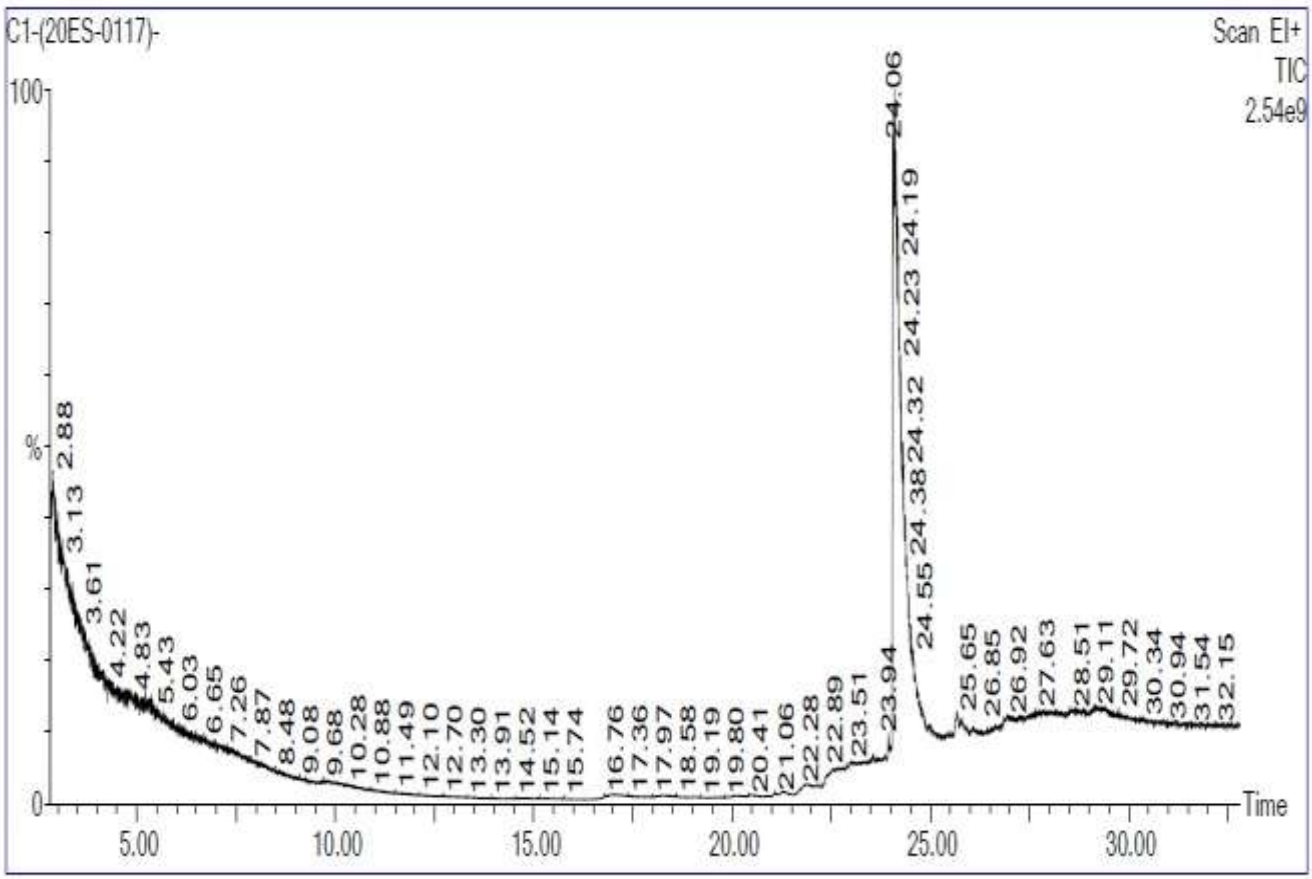

Figure 2: GC-MS chromatogram of Olive oil

Table 1
Compounds identified in methanolic extract of *Tridax procumbens* in GC-MS

S.N.	Name of the compound	Retention Time (R.T)	Molecular formula	Molecular weight	Peak %	Biological significance
1	Undecane	9.960	$C_{11}H_{24}$	156	0.46	Antimicrobial agent found in stem extract of *Solena amplexicaulis*[14]
2	Neophytadiene	19.507	$C_{20}H_{38}$	278	1.99	Good Anti-inflammatory, Antimicrobial and Antioxidant compound found in leaf extract of *Plectranthus amboinicus*[15]
3	2,6,10-trimethyl,14-ethylene-14-pentadecne	19.958	$C_{20}H_{38}$	278	0.68	Found in methanolic *Eupatorium triplinerve* extract acts as antiproliferative agent in cancer cells[16]
4	Benzene,(1methyldodecyl)-	20.317	$C_{19}H_{32}$	260	1.27	Bioactive compound present in *Calophyllum inophyllum*[17]
5	N-Hexadecanoic acid	20.765	$C_{16}H_{32}O_2$	256	0.96	Anti-inflammatory agent[18]
6	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-	22.226	$C_{20}H_{40}O$	296	0.92	Found in leaf and stem extract of *Marsilea minuta* and has Antioxidant and Antimicrobial[19]
7	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	22.472	$C_{42}H_{63}O_3P$	646	8.33	Inhibits quorum sensing mediated biofilm formation in pathogen[20]
8	Phenol,2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	22.671	$C_{42}H_{63}O_3P$	646	8.71	Inhibits quorum sensing mediated biofilm formation in pathogen[20]
9	Phenol,2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	22.756	$C_{42}H_{63}O_3P$	646	6.78	Inhibits quorum sensing mediated biofilm formation in pathogen[20]
10	Phenol,2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	22.858	$C_{42}H_{63}O_3P$	646	9.89	Inhibits quorum sensing mediated biofilm formation in pathogen[20]
11	Decane, 1-iodo-	23.455	$C_{10}H_{21}I$	268	1.76	No activity reported
12	Stigmasterol	23.520	$C_{29}H_{48}O$	412	2.07	Antioxidant, Anti-inflammatory and Antitumor activity[21, 22]
13	Stigmasterol	23.710	$C_{29}H_{48}O$	412	4.03	Antioxidant, Anti-inflammatory and Antitumor activity[21, 22]
14	Gamma.-sitosterol	26.167	$C_{29}H_{50}O$	414	3.37	Antihyperglycemic and Anti cancer activity[23]
15	Methyl commate A	26.240	$C_{32}H_{52}O_4$	500	1.04	Anti microbial activity[24]
16	Methyl commate A	27.210	$C_{32}H_{52}O_4$	500	12.21	Anti microbial activity[24]
17	Urs-12-en-3-ol, acetate, (3.beta.)-	27.665	$C_{32}H_{52}O_2$	468	1.56	Anti-inflammatory[25]
18	6.beta.Bicyclo[4.3.0] nonane, 5.beta.-iodomethyl-1.beta.-isopropenyl	27.792	$C_{15}H_{25}I$	332	0.69	Bioactive compound present in **essential oil of** *eugenia cotinifolia* **ssp**[26]
19	Stigmasterone	28.216	$C_{29}H_{46}O$	410	4.23	Present in plant extract of *Salvinia auriculata* Aubl which possess Antibacterial activity[27]
20	Methyl commate D	28.763	$C_{31}H_{50}O_4$	486	29.06	Presented in methanolic extract of *Eupatorium odoratum* which exhibits Antimicrobial and Anti-inflammatory process[28]

Table 2
Compounds identified in Olive oil of GC-MS

S.N.	Name of the compound	Retention Time (R.T)	Molecular formula	Molecular weight	Peak %	Biological significance
1	Squalene	24.097	$C_{30}H_{50}$	410	98.958	Antioxidant, Anticancer, antimicrobial and used in skin care regime[29]
2	Farnesol isomer A	24.787	$C_{15}H_{26}O$	222	1.042	Antibacterial, Antibiofilm and wound healing[30,31]

Antibacterial activity of *Tridax procumbens* extract, Olive oil and Herbal cream: All 3 different products namely *T. procumbens* methanolic leaf extract, olive oil and mixture of both as herbal cream shown as antibacterial activity against 4 different clinical pathogens (*E. coli, S. aureus, P. aeruginosa* and *K. pneumoniae*). Prepared herbal cream shows better antagonistic mechanism against clinical isolates rather than *T. procumbens* leaf extract and olive oil alone.

All the products were taken for well diffusion test individually with different concentration (20, 40, 60, 80 and 100 µg/mL) for each bacterial isolate. After incubation at 37 °C for 24 hour, the zone of inhibition were noted. Mean ± SD, n = 3 bacterial zone of inhibition rate was calculated statistically with $P < 0.05$ (Figure 3). Among the results 16.4 mm was the highest zone of inhibition found at *S. aureus* against 100 µg of herbal cream. Even a least concentration 20 µg of herbal cream shows 11-12 mm diameter zone for all 4 pathogens. This comparative analysis shows that prepared herbal cream has better antagonistic activity than plant extract and olive oil.

Antibacterial cream formulated for normal skin from *Mangifera indica was* found to have zone of inhibition of 10.2 mm, 8.9 mm for *E. coli* and *S. aureus* respectively[32]. Herbal ointment prepared from methanolic extract of *Cassia alata* shows inhibition rate 16.50 mm for *S. aureus* 50mg/mL and no zone for *E. coli* and *P. aeruginosa* which indicates our herbal cream preparation has great efficacy in antagonistic mechanism against clinical pathogens[33].

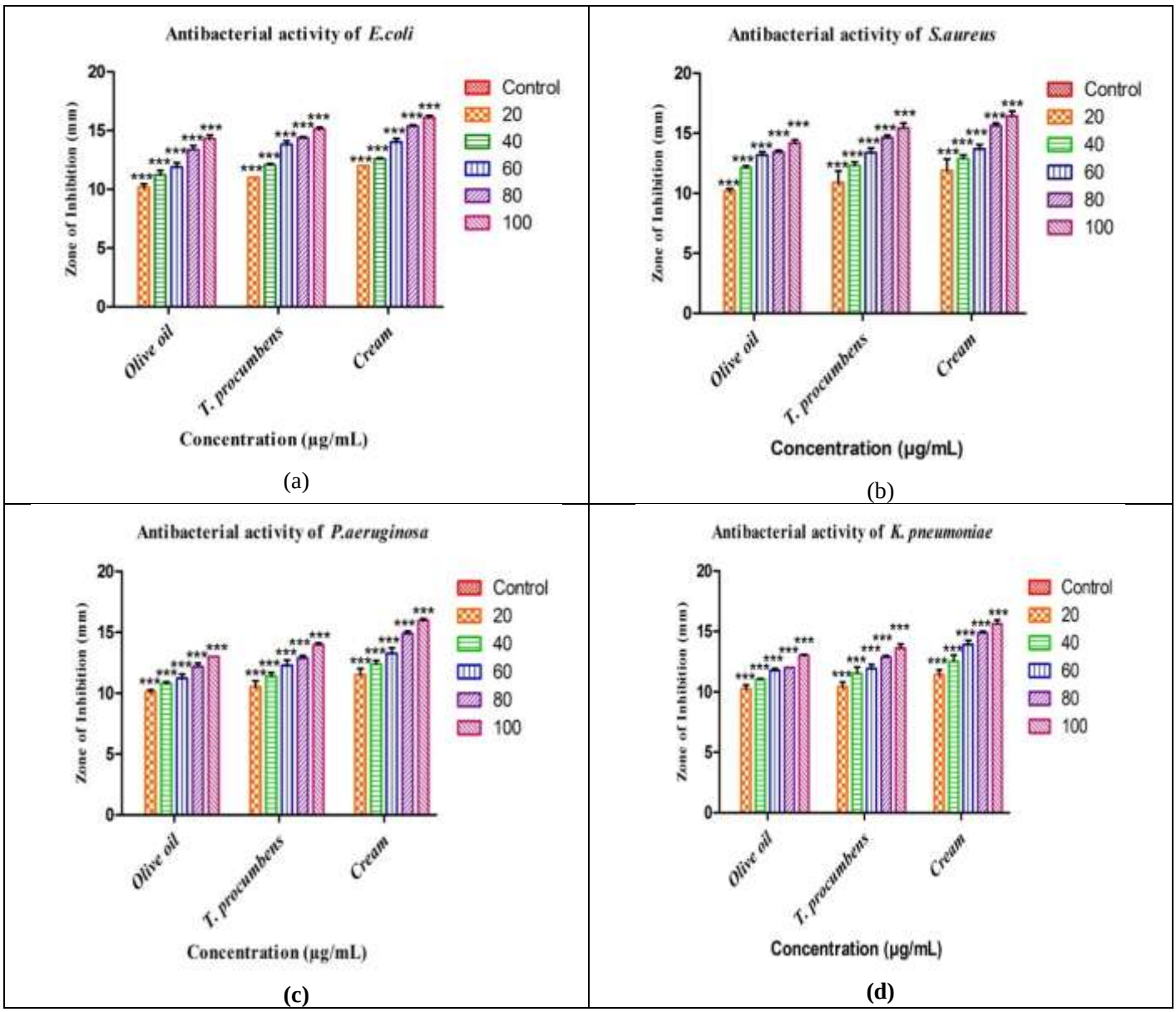

Figure 3: Antibacterial activity of methanolic extract of *Tridax procumbens*, Olive oil and Herbal cream on clinical pathogens by well diffusion method. The values were represented in Mean ± SD. * indicates the significant difference among the treatment with respect to control ($p < 0.001$).**

Antibioflim activity of *Tridax procumbens* extract, Olive oil and Herbal cream: Pathogenic biofilm reduction of 4 clinical isolates as listed above were studied using microtitre plate incubated with different concentration (20, 40, 60, 80 and 100 µg/mL) of *T. procumbens* methanolic extract, olive oil and herbal cream. The optical density of crystal violet bound to biofilm dissolved with 33 % of acetic acid shows inhibition capacity of the above mentioned products with respective concentration. The prepared herbal cream shows greater reduction potential than the plant *T. procumbens* extract and olive oil tested alone. The highest biofilm reduction rates for 4 clinical isolates namely *E. coli, S. aureus, P. aeruginosa* and *K. pneumoniae* at100 µg/mL were 33, 48.7, 39.7 and 34.3% for Olive oil, 41.4, 60.6, 55 and 47.3% for *T. procumbens* methanolic extract and 56.8, 66.6, 59.7 and 54.1% for herbal cream respectively (Figure 4).

Previous report of *T. procumbens* plant extract shows 41.9% of biofilm inhibition rate at100 µg/mL for *E. coli* [34]. 200 µg/mL of gentamicin shows 83, 75 and 96% of biofilm inhibition on *E. coli, P. aeruginosa* and *S. aureus* these inhibition rates were relatively lower than 100 µg/mL of herbal cream[35].

Hemolytic assay of *Tridax procumbens* extract, Olive oil and Herbal creams: Methanolic extract of *T.procumbens* shows higher hemolytic activity than prepared herbal cream (Figure 5). The hemolysis percentage in different concentration of *T. procumbens* methanolic extract, olive oil and herbal cream (20, 40, 60, 80 and 100 µg/mL) against 100 µL of RBC were noted. 0.7 and 1.8 % for 80 µg and 100 µg/mL of methanolic *T. procumbens* extract were observed. Olive oil does not show hemolytic activity on RBC for any respective concentration. 0.06% for 100 µg/mL was the maximum hemolysis observed for prepared herbal cream.

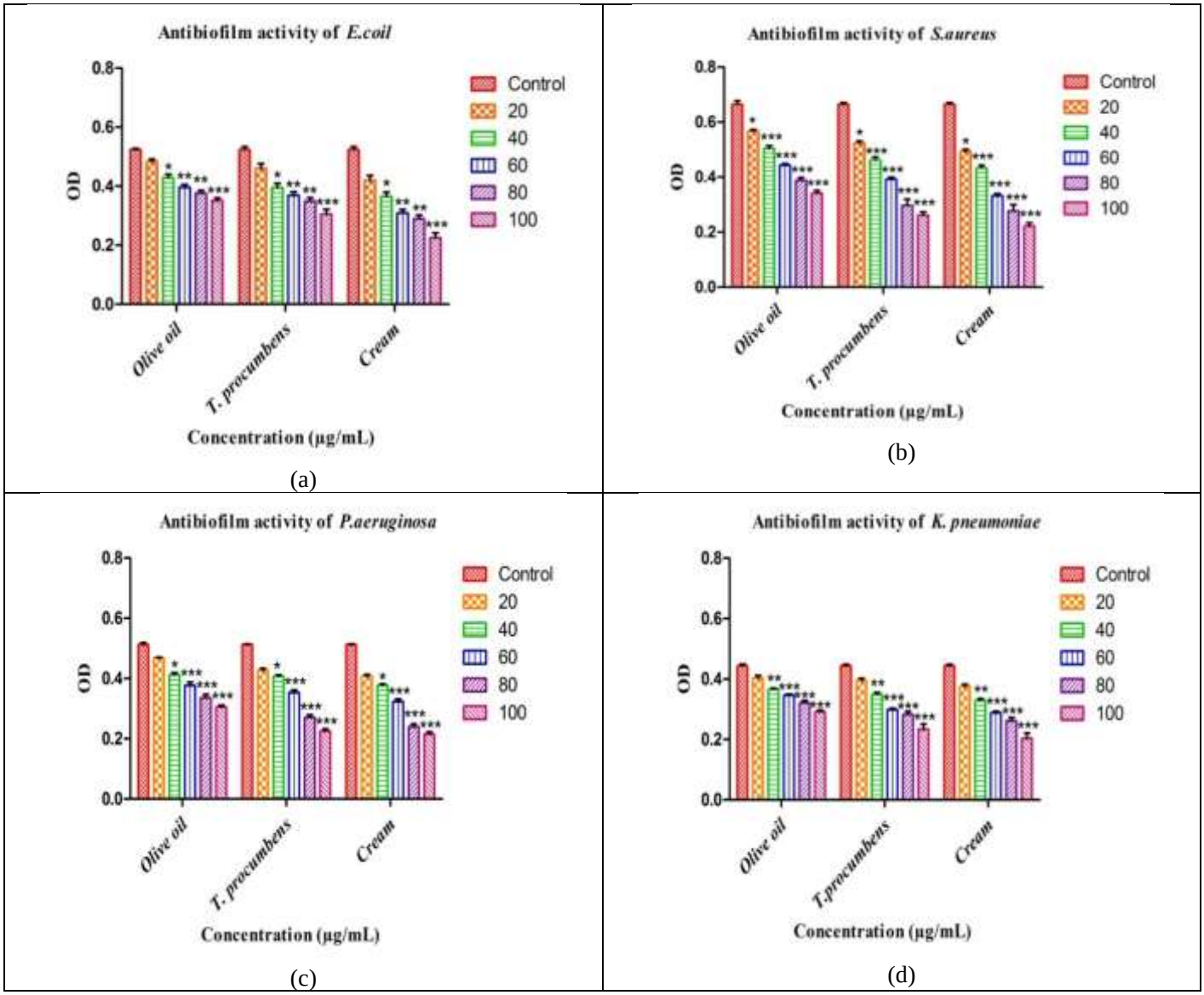

Figure 4: Antibiofilm potential of clinical isolates at OD_{550nm} in response to methanolic extract of *Tridax procumbens*, Olive oil and Herbal cream. The values were represented in Mean ± SD. *, ** and * indicates the significant difference among the treatment with respect to control ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$).**

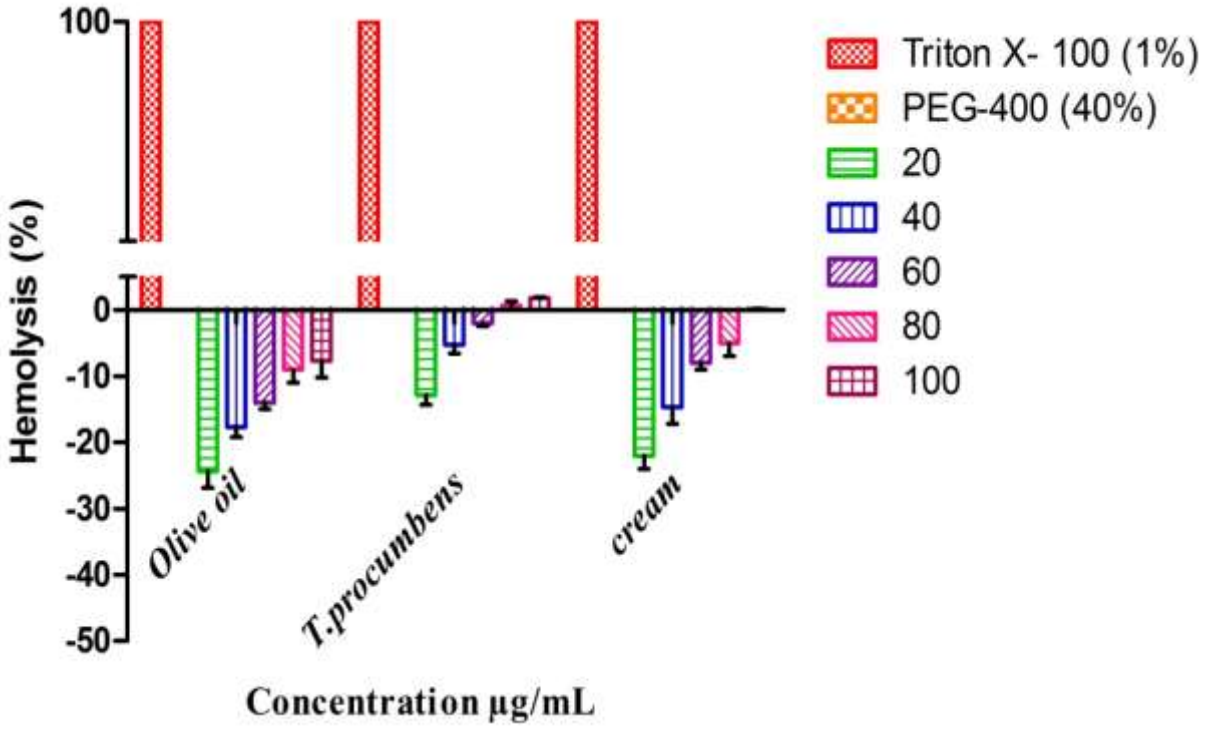

Figure 5: Hemolytic assay using different concentration of methanolic extract of *Tridax procumbens*, Olive oil and Herbal cream. Triton X-100 (1%) and PEG-400 (40%) were used as positive and negative control respectively. The values were represented in Mean ± SD, n = 3. The significant difference among the treatment with respect to control was ($p < 0.001$).

In early reports 100 µg/mL *Paronychia chlorothyrsa* plant extract shows 0.098% hemolysis which is slightly equal to prepared herbal cream[36]. Thus this study confirms combination of olive oil with plant extract for antibiotic cream preparation with low hemolytic effect on the product.

Conclusion

The study indicates herbal cream prepared from methanolic extract of *Tridax procumbens* and olive oil exhibits stronger antibacterial activity against clinical pathogens and also possesses low hemolytic activity on human erythrocytes. Thus the prepared *Tridax procumbens* herbal cream can be used topically on wounds to reduce the destruction caused by pathogens.

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