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SAFETY PROFILE AND TOXICOLOGICAL EVALUATION OF ESSENTIAL OILS OF CURCUMA LONGA AND SYZYGIUM AROMATICUM

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ABSTRACT
Medicinal plants are the major source of natural organic compounds and are universally used as alternative medicines. Essential oils of *Curcuma longa* (turmeric) and S*yzygium aromaticum (Clove)* have anti-inflammatory, antioxidant, analgesic, anti-rheumatic, and antimicrobial properties. However, if essential oils of these plants have to be used as therapeutic agents, then their safety and toxicity also need to be addressed. In the present study, the safety profile of essential oils of S*yzygium aromaticum* and *Curcuma longa* were evaluated by Comet assay, MTT assay, and AMES test. Both essential oils exhibited good tolerability and safety profiles at a concentration of 50 µL/mL that was quite higher than the MICs observed against different multidrug-resistant pathogenic bacteria. The IC-50 values observed were 58 and 62.79 for S*yzygium aromaticum* and *Curcuma longa* respectively. It was concluded that the essential oils of S*yzygium aromaticum* and *Curcuma longa* have a good safety profile and can be used for medicinal purposes.

Keywords: Essential oil, *Curcuma longa,* S*yzygium aromaticum,* MTT Assay, AMES test, Comet Assay

INTRODUCTION
For thousands of years, nature has been facilitating human beings by providing medicinal agents and a magnificent number of the latest drugs have been isolated from plants (Cragg and Newman 2002). According to the World Health Organization, almost 80% of the world's population depends on conventional medicines for their primary healthcare needs. It has been observed that there are significant productive benefits in the progression of local medicines and in the use of indigenous medicinal plants to treat various diseases (Azaizeh et al. 2003).
Essential oils derived from different medicinal plants are volatile and consist of different chemical compounds with vigorous aroma. Different methods of extraction e.g. hydro distillation, steam distillation, and solvent extraction, have been developed and progressed in the Middle Ages by

Arabs (Bakkali et al. 2008a; Raut and Karuppayil 2014). Essential oils and their chemicals can be used as an alternative to the synthetic compounds being used in the chemical, food, and medicinal industry, due to less harmful side effects (Carson and Hammer 2011). Extracts and essential oils from different medicinal plants possess antimicrobial activities. However, if these essential oils are used for medicinal use and as food preservatives then issues of safety and toxicity need to be addressed carefully (Hammer et al. 1999). Syzygium aromaticum has antibacterial qualities due to eugenol, which inhibits bacteria and fungi and may have antiviral activities. It also has anti-inflammatory benefits by lowering oxidative stress and decreasing mediators (Tabassum, Anjum et al. 2022, Utami, Wahyuni et al. 2023). The primary ingredient in turmeric (Curcuma longa), curcumin, has antibacterial, antifungal, and maybe antiviral qualities. It also has anti-inflammatory effects via modifying pathways and lowering oxidative stress (Anwar, Yasmeen et al. 2023, Iweala, Uche et al. 2023). Many recent researches conducted on different plant extracts reported their strong anti-microbial potential but very low safety profile and high toxic potential (Hayes and Markovic 2002; Martini et al. 2004). This study evaluated two medicinal plants essential oils for their safety profiles by genotoxic, cytotoxic, and mutagenic properties using Comet assay, MTT assay, and Ames test, respectively.

MATERIALS AND METHODS

Essential oils of *Syzygium aromaticum* and *Curcuma longa* were extracted by steam distillation, using industrial scale distillation assembly, and their antimicrobial activity was assessed by calculated MIC values against multiple drug-resistant bacteria (Zeshan et al. 2023). The active crude plant essential oils were evaluated for their safety profile.

Evaluation of genotoxicity by Comet assay

The genotoxicity of the selected essential oils was evaluated by performing a Single cell gel electrophoresis assay generally called as Comet assay. The DNA Damage was evaluated at different concentrations (2000µL/mL, 1000µL/mL, 800µL/mL, 600µL/mL, 400µL/mL, 200µL/mL, 100µL/mL and 50µL/mL) of the tested essential oils. The cavity slides were prepared by dipping into methanol and were influenced under the flame to remove oily and greasy particles. The slides were dipped into normal melting agarose to fill the cavities and dried by placing a horizontal surface. After drying sliders were stored for 4 to 12 hours in the refrigerator. For the cell isolation 3 mL of blood was mixed with 3 mL PBS then 4 mL of this mixture solution was taken and mixed with lymphocyte separating medium and centrifuged at 800rpm for 45 minutes. Lymphocytes were removed and again treated with PBS and centrifuged at 250rpm for 10-15 minutes.

Cell suspension of the lymphocytes was treated with different concentrations of the essential oils in the separated eppendorf tube and incubated for 2 hours. The tubes were centrifuged at 3000rpm to obtain the lymphocyte pallet. Cell suspension was treated with essential oils and low melting points agarose solution poured onto the slide's cavities and treated with lysine solution in a dark place for 12 hours. Then, treated with alkaline buffers and gel slides were electrophorized at 24 volts. The slides were stained with Ethidium bromide and observed under a florescent microscope for DNA damage by using J launcher Image software (Parolini et al. 2009). RPMI medium was used as the negative control and 20% DMSO as the positive control. The cells were divided into four groups to evaluate comet scoring. The number of cells in each class was 25. Genetic Damage index (GDI) was calculated following (Kousar and Javed 2015).

$$GDI = \frac{(1\times \text{No. of cells in class-1}) + (2 \times \text{No. of Cells in Class-2}) + (3\times \text{No. of Cells in class-3})}{\text{No. of Cells in class-0} + \text{No. of Cells in class-1} + \text{No. of Cell in class-2} + \text{No. of cells in class 3}}$$

Evaluation of cytotoxic properties by MTT Assay

To evaluate the cytotoxic potential of essential oils, an MTT assay was performed as per protocols described by (Sabová et al. 2010). Cell line (BHK-21) was treated with different dilutions of essential oils in a 96-well plate with a flat bottom. Stock solutions of essential oils were prepared

with different concentrations ranging from 0.39µL/ mL to 2000µL/ mL in 1% Dimethyl sulphoxide (DMSO). Each concentration was tested by pouring in triplicate. After closing the plate lids, these were incubated in a CO_2 incubator for 48 to 72 hours at 37°C. After incubation, the cell culture medium was discarded and cells were treated with 20µL of MTT and 100µL of fresh medium was added in each well and incubated on the plate for a further 3 to 4 hours. After the incubation period medium was removed and treated cells with 100µL of DMSO and the optical density of each well was recorded by an ELISA reader. By taking the cell culture medium as positive control and DMSO as negative control, Cell survival percentages were calculated and by plotting on a graph pad prism IC-50 values were calculated. The experiment was performed thrice for each concentration and the mean OD value was used to calculate the Cell survival percentage.

$$\text{Cell Survival Percentage (CSP)} = \frac{\text{Mean OD of tested Solution} - \text{Mean OD of negative control}}{\text{Mean OD Value of positive Control}} \times 100$$

Evaluation of mutagenicity of essential oils by Ames test

The mutagenic potential of essential oils was evaluated by employing the bacterial reverse mutagenic assay, commonly known as the Ames test. The muta-chrome plate was used for the Ames test. Broth cultures of *Salmonella typhimurium* TA-98 and *Salmonella typhimurium* TA-100 were used to perform this test. The tested essential oil, reagent mixture, Salmonella activation mixtures, standard mutagen, and bacterial broth cultures were mixed in several bottles separately as described in manual kits. Contents from all bottles were dispensed in 96-well plates and incubated for four days.

The background plate contained a reagent Mixture (2.5 mL), Sterilized deionized water (17.5 mL), and test strain (0.005mL).

The standard plate contained Standard mutagen (0.1mL) regent Mixture (2.5 mL), Sterilized deionized water (17.5mL), and test strain (0.005mL).

Test Plate contained essential oil (0.01mL), reagent Mixture (2.5 mL), Sterilized deionized water (17.5mL), and test strain (0.005mL).

Yellow-colored wells that appeared after incubation were denoted as positive while purple colored were considered as negative (Gilbert 1980). Results were interpreted by calculating the mutagenic index as given formula:

$$\text{M.I.} = \frac{\text{Number of Revertant colonies per plate with test chemical dose}}{\text{Number of Revertant colonies of negative control plate}}$$

The essential oils showing M.I. value less than 2 were declared as non-mutagenic, greater than 2 as mutagenic, and greater than 3 as significant mutagenic.

Statistical analysis of results

The results were analyzed through a statistical package for social sciences (SPSS) using one-way ANOVA, post hoc Duncan, and the Chi-square model was used.

Results

The MIC valves observed against different multiple drug-resistant bacteria are given in Table 1 (Zeshan et al. 2023)

The mean tail length and GDI were observed at the various concentrations of the tested essential oils i.e. 2000 µL/mL, 1000 µL/mL, 800 µL/mL, 600 µL/mL, 400 µL/mL, 200 µL/mL, 100 µL/mL and 50 µL/mL. For *Syzygium aromaticum* essential oil (Table 2) mean tail length observed at the highest concentration i.e., 2000 µL/mL was 0.99±0.37 µm, and GDI was 1.04, which was lesser than 13.35±0.01 µm and 2.12 observed for positive control. For *Curcuma longa* essential oil (Table 3), the mean tail length at the highest concentration, i.e. 2000 µL/mL was 1.41±0.32µm and GDI was

1.16 that was lesser than 13.35±0.01µm and 2.12 observed for positive control. The results were statistically significant (P≤0.05) which indicated that all tested concentrations were safer as these were higher than MIC values already reported in previously published work (Table 1).

Table1: Minimum Inhibitory Concentrations (µL/mL) of essential oils of *S. aromaticum* and *C. longa* (Zeshan et al. 2023)

Essential Oils	MIC (µL/mL) of essential oils of *S. aromaticum* and *C. longa*			
	S. aureus (MRSA)	*K. pneumonia*	*E. coli*	*A. baumannaii*
S. aromaticum	1.69 ±0.76	2.06±0.08	3.12±1.17	1.04±0.42
C. longa	37.5±13.69	47.92±30.17	41.67±12.19	31.25±15.30

Table 2:Mean length of head and tail of damaged DNA at various concentrations of *Syzygium aromaticum* essential oil.

Concentration (µL/mL)	Mean±S.D. head length (µm)	Mean±S.D. tail length (µm)	Head-to-tail ratio	Class-0	Class-1	Class-2	Class-3	Damaged index	GDI
2000	2.05±0.62	0.99±0.37	2.07	0	24	1	0	26	1.04
1000	2.14±0.32	0.67±0.24	3.19	0	25	0	0	25	1.00
800	2.18±1.05	0.61±0.22	3.57	1	24	0	0	24	0.96
600	2.21±0.61	0.35±0.43	6.31	6	19	0	0	19	0.76
400	2.29±0.58	0.25±0.23	9.16	9	16	0	0	16	0.64
200	2.46±0.51	0.24±0.22	10.25	12	13	0	0	13	0.52
100	2.69±0.75	0.18±0.26	14.94	14	11	0	0	11	0.44
50	3.21±1.19	0.13±0.22	24.69	19	9	0	0	9	0.36
20% DMSO (Positive Control)	0.78 ±0.01	13.35±0.01	0.06	0	10	2	13	53	2.12
RPMI (Negative Control)	5.78 ±0.16	0.13 ±0.01	44.46	23	2	0	0	2	0.08

Class 0: Represent undamaged cells.
Class 1: Represent cells with tail lengths less than or equal to the head diameter.
Class 2: Represent cells with a tail length more than the head diameter but less than double the diameter of the head.
Class 3: Represents cells with tail length greater than double the diameter of the head.
GDI: Genetic Damage index
S.D.: Standard deviation

Table 3 Mean length of head and tail of damaged DNA at various concentrations of *Curcuma longa* essential oil.

Concentrations (µL/mL)	Mean±S.D. head length (µm)	Mean±S.D. tail length (µm)	Head-to-tail ratio	Class-0	Class-1	Class-2	Class-3	Damaged index	GDI
2000	1.95±0.51	1.41±0.32	1.38	-	21	4	-	29	1.16
1000	2.13±0.52	1.22±0.42	1.75	-	23	2	-	27	1.08
800	2.23±0.36	1.18±0.36	1.89	-	24	1	-	26	1.04
600	2.37±0.39	1.05±0.42	2.26	-	25	-	-	25	1
400	2.6±0.29	0.86±0.39	3.02	-	25	-	-	25	1
200	2.71±0.41	0.75±0.32	3.61	2	23	-	-	23	0.92
100	2.9±0.32	0.68±0.22	4.26	3	22	-	-	22	0.88
50	3.37±0.45	0.52±0.27	6.48	5	20	-	-	20	0.8

20% DMSO (Positive Control)	**0.78 ±0.01**	**13.35±0.01**	**0.06**	**0**	**10**	**2**	**13**	**53**	**2.12**
RPMI (Negative Control)	**5.78 ±0.16**	**0.13 ±0.01**	**44.46**	**23**	**2**	**0**	**0**	**2**	**0.08**

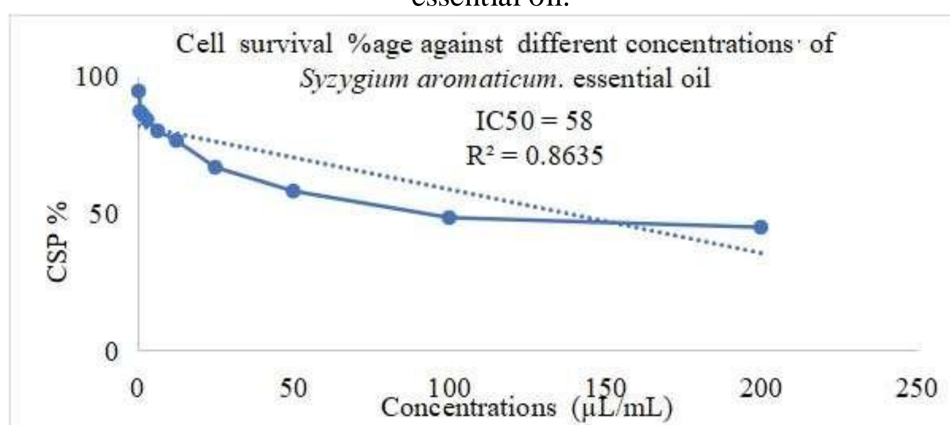
Class 0: Represent undamaged cells.
Class 1: Represent cells with tail lengths less than or equal to the head diameter.
Class 2: Represent cells with a tail length more than the head diameter but less than double the diameter of the head.
Class 3: Represents cells with tail length greater than double the diameter of the head.
GDI: Genetic Damage index
S.D.: Standard deviation

Table 4. Mean Optical density (OD) and Cell survival percentage (CSP) at various concentrations of *Syzygium aromaticum* and *Curcuma longa*

Concentrations (µL/mL)	***Syzygium aromaticum***		***Curcuma longa***	
	Mean OD±SD	**Cell survival percentage (CSP)**	**Mean OD±SD**	**Cell survival percentage (CSP)**
200	0.279±0.049	45	0.277±0.001	35.2
100	0.291±0.013	48.5	0.313±0.001	42.6
50	0.331±0.008	58.2	0.354±0.001	51.0
25	0.368±0.013	66.8	0.386±0.021	57.6
12.5	0.408±0.047	76.7	0.423±0.027	65.13
6.25	0.423±0.018	80.2	0.463±0.009	73.33
3.13	0.441±0.070	84.3	0.475±0.052	75.74
1.56	0.449±0.034	86.3	0.494±0.047	79.67
0.78	0.453±0.009	87.3	0.516±0.016	84.36
0.39	0.482±0.018	94.7	0.543±0.017	89.82
100 DMSO (Negative control)	0.090±0.004		0.105±0.010	
Cell culture medium (Positive control)	0.415±0.005		0.488±0.013	

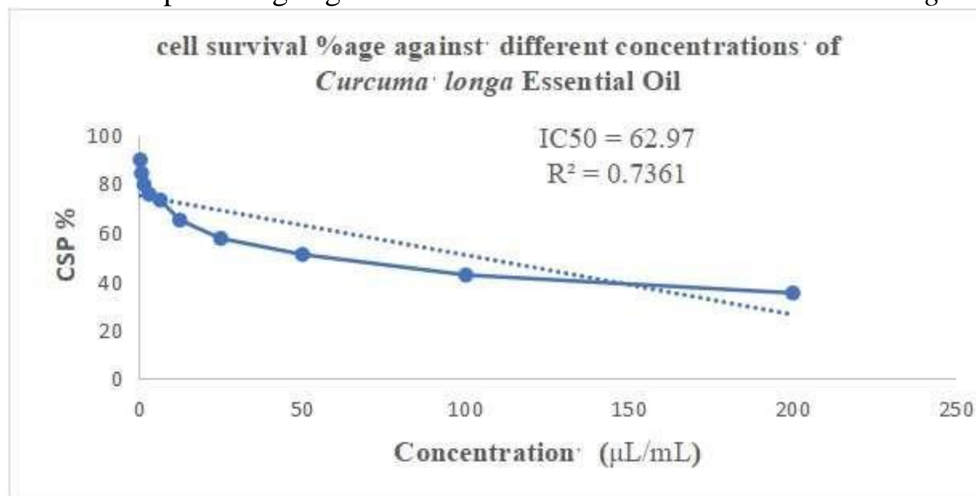
Cell survival percentage (CSP%) of tested essential oil was evaluated at concentrations 200µl/ml, 100 µl/ml, 50 µl/ml, 25µl/ml, 12µl/ml, 6.25 µl/ml, 3.13µl/ml, 1.56µl/ml, 0.78µl/ml, and 0.39µl/ml respectively. The cell survival percentage was more than 50% at the concentrations of 50 µL/mL and less than 50% at the concentrations of 100 and 200 µL/mL for both essential oils i.e. *Syzygium aromaticum* and *Curcuma longa*, (Table 4). Exact IC50 values were calculated as 58 and 62.97 for *Syzygium aromaticum* and *Curcuma longa* respectively (Figure 1 and Figure 2) by plotting the graph between concentrations of essential oils (x-axis) and cell survival percentage (on Y-axis).

Figure No. 1 Cell survival percentage against different concentrations of *Syzygium aromaticum* essential oil.



CSP= Cell survival percentage

Fig. No.2 Cell survival percentage against different concentrations of *Curcuma longa* essential oil.



CSP= Cell survival percentage

Two strains of *Salmonella typhimurium* TA98 and TA100 were used as test organisms. The number of reverting colonies observed for ***Syzygium aromaticum*** **and** ***Curcuma longa*** were 16 and 20 for TA-98 and 12 and 25 for TA-100 respectively. While 90 and 86 reverting colonies were observed for K2Cr2O (standard for TA-98) and NaN3 (standard for TA-98) respectively. The number of reverting colonies observed for media (positive control) were 14 and 27 for TA-98 and TA-100 respectively (Table 6). MI values calculated for ***Syzygium aromaticum*** were 1.14 and 0.04 and for ***Curcuma longa*** were 1.42 and 0.92 respectively. The value of the mutagenic index was less than 2 which indicated that tested essential oils were non mutagenic

Table 6: Ames assay for essential oils of *Syzygium aromaticum* and *Curcuma longa*

Samples	TA-98	Results	TA-100	Results
Syzygium aromaticum	16/96	Non-mutagenic	12/96	Non-mutagenic
Curcuma longa	20/96	Non mutagenic	25/96	Non mutagenic
Standard (K2Cr2O7)for TA-98 and (NaN3 for TA-100) (Positive Control)	90/96	Mutagenic	86/96	Mutagenic
Background Negative Control (media)	14/96	Non-mutagenic	27/96	Non-mutagenic
M.I of *Syzygium aromaticum*	16/14= 1.14	Non-mutagenic	12/27 = 0.44	Non-mutagenic
M.I of *Curcuma longa*	20/14 = 1.42	Non-mutagenic	25/27 = 0.92	Non-mutagenic

M.I = mutagenic index

DISCUSSION

Medicinal plants not only provide an effective, and tolerable therapeutic tool in the form of the drug but also ensure economic benefits for society as traditional drugs are more economical than synthetic drugs (Anwar et al. 2023; Javed et al. 2023; Azaizeh et al. 2003). The use of *Syzygium aromaticum* and Curcuma longa as a medicine, food preservative, and spice is established by several studies available in the literature (Saeed et al. 2013, Krup et al. 2013). *Syzygium aromaticum is* well known for its anti-inflammatory, analgesic, antifungal, antithrombotic, and anticarcinogenic properties (Mittal et al. 2014). Assessing the safety profile and tolerability along with the potency

and efficacy of the traditional drugs is equally important. As an alternative, a traditional drug should have the same efficacy as the conventional drug with fewer side effects. Many plant extracts showed excellent antimicrobial efficacy but cytotoxicity screening reveals toxic effects against human lymphocytes and cell lines of liver and skin (Hayes and Markovic 2002; Martini et al. 2004). An attempt has been made to study the safety profile of essential oils of *Syzygium aromaticum* and *Curcuma longa* by evaluating their Cytotoxic, mutagenic, and DNA-damaging properties by performing MTT, Ames, and Comet assays. The tested concentrations of essential oils of *Syzygium aromaticum and Curcuma longa* did not damage DNA as indicated by the genetic damage index. Different researchers also reported good safety profiles which correlate with the present research (Elzayyat et al. 2018; Jahangir et al. 2020; Liju et al. 2013). The IC50 values recorded for *Syzygium aromaticum* was 58µL/mL, which was higher than the MIC values of its essential oil against different multiple drug-resistant strains of methicillin-resistant *Staphylococcus aureus, Klebsiella· pneumoniae, Acinetobacter baumannii* and *Escherichia coli*. Different researchers have reported the IC-50 value of *Syzygium aromaticum* ranging from 15.75 to 200 µL/mL (Behbahani et al.; 2019: Kaur and Kaushal 2019; Kouidhi et al. 2010). This variation in IC50 may be due to variation in the cell line used for the experiment and method of extraction of essential oil. In the case of *Curcuma longa* IC-50 value, calculated as 62.97 µL/mL which was higher than MIC values observed against different multiple drug-resistant strains of methicillin-resistant *Staphylococcus aureus, Klebsiella· pneumoniae, Acinetobacter, baumannii, and Escherichia coli* re. IC-50 value reported in the literature for *Curcuma longa* correlates with the present study(Sadashiva et al. 2019; Santos et al. 2016). The results indicated that tested essential oils were safe at their minimum antibacterial concentration.

For *Syzygium aromaticum* and *Curcuma longa* essential oils, non-mutagenic property was observed as the mutagenic index (MI) values were less than 2. Different researchers reported the non-mutagenic properties of *Syzygium aromaticum* even at the high dose which correlates with the present study. Further, some researcher reports its anti-mutagenic properties thus it can be easily revealed that essential oil can be used as an antibacterial agent (Mittal et al. 2014; Vijayasteltar et al. 2016).

Similarly, the safety profile of *Curcuma longa* oil has also been reported by different researchers (Liju et al. 2013; Velusami et al. 2013). It was concluded that essential oils of *Syzygium aromaticum* and *Curcuma longa* (50µL/mL) were safe for use against multiple drug-resistant bacterial infections *in vitro*. Therefore, there may be *in-vivo* trials for use in animals and human beings as an alternative to antibiotics.

Conclusion:

The study's findings highlighted the potential of essential oils derived from Syzygium aromaticum and Curcuma longa as antibiotic substitutes by demonstrating their safety profile. Below the minimal inhibitory concentrations against drug-resistant bacteria, the assessed concentrations demonstrated no cytotoxicity, no DNA damage, and no mutagenic characteristics. The safety of both essential oils at antibacterial concentrations was confirmed by the fact that their IC50 values were higher than their MIC values. These results encourage more investigation into these essential oils as viable candidates for in vivo tests to treat bacterial infections.

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Author's contribution:

Muhammad Qamar Zeshan and Muhammad Ashraf, Conceptualization, Data Curation, Writing, Formal analysis, Project Administration; Muhammad Ovais Omer, Investigation; Muhammad Ashraf, Aftab Ahmad Anjum, and Muhammad Asad Ali, Methodology, Resources, Data Curation; Supervision, Validation, Visualization; Muhammad Qamar Zeshan Writing, Original draft; Shagufta Yasmin, and Javeria Majeed, Data Curation, Validation, Visualization; Muhammad Mubashar Beig, Review, Writing . All the respective authors did read through the manuscript and then agreed that it should be published

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