ORIGINAL ARTICLE

# Interference from ordinarily used solvents in the outcomes of *Artemia salina* lethality test

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## ABSTRACT

Methanol, ethanol, Tween 20 and dimethyl sulfoxide (DMSO) are widely used as dissolving agents in *Artemia salina* lethality test (aka brine shrimp lethality test [BSLT]) to screen the pharmaceutical properties of natural products. Nevertheless, there is lack of toxicity level of these solvents against brine shrimp. High concentration of these organic solvent might be toxic for this zoology invertebrate and interfere in the experimental outcomes. To avoid this, permissible concentration of the solvents used in BSLT was identified. BSLT was performed to evaluate the toxicity effect of Tween 20, methanol, ethanol and DMSO at 24 h post-treatment time point against *A. salina*. The suggested maximum working concentration (v/v) for DMSO, methanol, ethanol was found to be 1.25% and that for Tween 20 was 0.16%. $LC_{50}$ for the solvents were 8.5% (DMSO), 6.4% (methanol), 3.4% (ethanol) and 2.5% (Tween 20). The findings have shown a toxicity level among the solvents in descending order as Tween 20 > ethanol > methanol > DMSO. DMSO is a safer solvent to be used in BSLT compared with other tested solvents, whereas Tween 20 has been shown to be the most stringent solvent among the tested solvents. The findings are resourcefully useful to avoid interference of solvents in the assessment of natural products using BSLT.

**Key words:** Brine shrimp, brine shrimp lethality test, dimethyl sulfoxide, ethanol, methanol, Tween 20

## INTRODUCTION

Brine shrimp lethality test (BSLT) is a broad spectrum bioassay capable of detecting cytotoxicity effect and bioactive presence in an extract. It is an easily performed, cost-effective test. Brine shrimp or scientifically known as *Artemia salina* is a simple zoology invertebrate organism.[1] There are several advantages using BSLT in pharmacological studies. McLaughlin and Lagarto found a positive correlation between toxicity against brine shrimp and cytotoxicity against 9KB (human nasopharyngeal carcinoma) ($P$ = 0.036 and kappa = 0.56), as well as to the median lethal concentration ($LC_{50}$) of the acute oral toxicity assay in mouse ($r$ = 0.85; $P$ < 0.05).[1-3] BSLT was also reported as a tool for the evaluation of cytotoxicity and pesticide activity of compounds and was considered to be a very useful preliminary tool for isolation of bioactive compounds from plant extracts.[4] Numerous scientific works have been conducted using BSLT for determining the toxicity effect of compounds from different origins, such as the works conducted on *Meliaceae* family medicinal plants,[5] Savannah plants,[6] marine natural products,[7] some species of *Solanum* from North-eastern Brazil[8] and *Terminalia brownii* roots and stem extracts.[9] The test materials were dissolved in various solvents for testing. In general used solvents include methanol, dimethyl sulfoxide (DMSO), Tween 20, water and ethanol. Each of the solvents has its own toxicity properties. However, there is no scientific evidence of their toxicity effect on brine shrimp being reported thus far. As it is crucial to prevent the toxicity of a solvent from interfering in BSLT, the present study was focused on the evaluation of the toxic effect induced by the commonly used solvents at various concentrations on *A. salina*.

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## MATERIALS AND METHODS

### Solvents

DMSO, ethanol, methanol AR grade (all from Qrec, Malaysia) and Tween 20 (Sigma-Aldrich, USA) were used in this test.

### Hatching of brine shrimp

The procedure for BSLT was modified from the assay described by McLaughlin and Rogers.[1] Brine shrimp (*A. salina*) eggs (Sanders™ Great Salt Lake, Brine Shrimp Company L.C., U.S.A.) were hatched in artificial sea water prepared from commercial sea salt (38 g sea salt/L deionized water) with constant light source and oxygen supply after 24 h of incubation. 48 h old nauplii were used for bioassay.

### Bioassay

Methanol, DMSO, ethanol and Tween 20 were used in the study to determine the toxicity effect. The test materials were freshly prepared and two-fold serial dilution technique in salt water was carried out in the range of 0.3125-20% (v/v). The phototropic nauplii were collected with pipette and ten 48 h old nauplii were transferred to each well and incubated for 24 h. Each concentration, including positive control group (potassium dichromate [$K_2Cr_2O_7$]) and negative control groups (phosphate buffer saline and artificial sea water), had three replicates. The toxicity was estimated by $LC_{50}$ determined after 24 h incubation period (concentration of the solvent with 50% of the test animals killed after 24 h and exposure). The nauplii were considered as dead if no movement was detected during the 10 s observation period. The mortality of the brine shrimp was calculated using the formula:

Mortality rate (%) = (death nauplii/total nauplii) × 100%

The $LC_{50}$ values were calculated by graphics from concentration versus lethality percentage using Probit analysis[5] on a Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada). Percentage mortalities were corrected for the natural mortality observed in the negative controls using Abbott's formula, $P = (p_i - C)/(1 - C)$, where $p_i$ denotes the observed mortality rate and C means the natural mortality. Each solvent concentration was replicated three times.

## RESULTS

Figure 1 shows the number of dead *A. salina* with the increment of concentrations of the tested solvents. Mortality of *A. salina* was first observed at 2.5% of DMSO and methanol, at 1.25% of ethanol and at 0.3125% of Tween 20. As concentration of solvents increased, the shrimps were eventually killed totally. This was found to occur at 2.5% of ethanol and Tween 20, at 5% of methanol and at 10% of DMSO. The toxicity level of the tested solvents in descending order was therefore deduced as Tween 20 > ethanol > methanol > DMSO. The respective $LC_{50}$ values calculated using Probit analysis[5] were shown in Table 1. From the above findings, maximum tolerable concentrations were suggested for BSLT conducted using the tested solvents and were shown in Table 1. The suggested maximum tolerable concentrations were 1.25% for DMSO, methanol and ethanol and 0.16% for Tween 20. $K_2Cr_2O_7$ is typically used as positive control in BSLT assay. *A. salina* was killed starting from 6.25 µg/ml concentration. The negative control represents an appropriate growth media for *A. salina.*

## DISCUSSION

The importance of BSLT has been evidenced in the determination of bio-activity of synthetic or natural plant products,[8] detection of anti-tumor compounds in terrestrial plant extracts[10,11] and *in vitro* growth inhibition of human solid tumor cell lines.[12] Nevertheless, there is a lack of uniformity in the concentration used for these solvents in pharmacological evaluation. This study has identified the limiting concentration of the solvents that could be used for BSLT without the outcomes of the test being affected by the solvents.

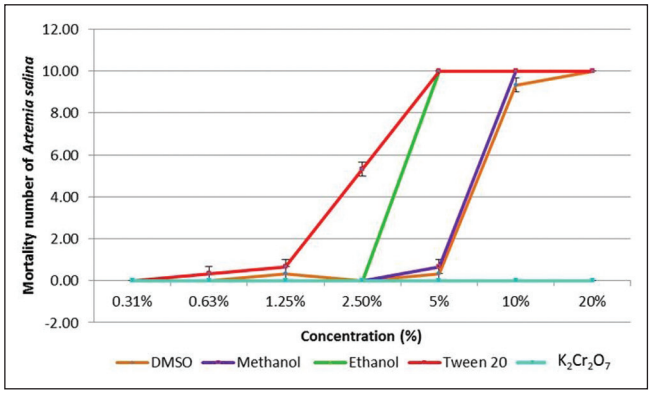


**Figure 1:** The number of dead *Artemia salina* at various percentage of dimethyl sulfoxide, methanol, ethanol, Tween 20 and $K_2Cr_2O_7$ respectively in salt water ($n = 3$)

**Table 1: The maximum tolerable concentrations and $LC_{50}$ values for various dissolving agents against *A. salina***

| Solvents | Maximum tolerable concentrations (%) | $LC_{50}$ (%) |
|---|---|---|
| DMSO | 1.25 | 8.5 |
| Methanol | 1.25 | 6.4 |
| Ethanol | 1.25 | 3.4 |
| Tween 20 | 0.16 | 2.5 |
| Potassium dichromate | <0.001* | <0.001** |

*Exact concentration is at 6.25 µg/ml, **Concentration at 11.35 µg/ml, DMSO: Dimethyl sulfoxide, *A. salina: Artemia salina*, LC: Lethal concentration

Brine shrimps were cultured in various solvents at various concentrations for 24 h. DMSO, methanol, ethanol and Tween 20 are commonly used solvents in bioassays such as BSLT, antimicrobial and cytotoxicity activity tests. DMSO is widely used to dissolve plant extracts. Similarly, methanol and ethanol are also extensively used to dissolve a large number of chemical constituents in natural products. Nevertheless, albumin, gums, waxes, sucrose, fats and fixed oils are insoluble in methanol and ethanol. Tween 20 is very useful in dissolving the essential oil and other oil substances in plant extracts.

Both methanol and DMSO were found to exert the killing effect on brine shrimp at 2.5%. However, the number of dead *A. salina* in methanol was found to be higher than DMSO at this concentration, indicating that DMSO might be less toxic compared with methanol at 2.5%. DMSO was therefore classified as the least toxic solvent, among the tested solvents, against brine shrimps.

Ethanol is well-known to be one of the best extractants to extract the phytoconstituents from plant materials.[13] Nevertheless, this organic solvent was found to have toxic effect against brine shrimps at 2.5%. All the tested brine shrimps were killed at 2.5% of ethanol, unlike that with methanol (5%) and DMSO (10%), suggesting that ethanol was more toxic than these latter.

Although all tested brine shrimps were killed at 2.5% of Tween 20, similar to that of ethanol, killing of the nauplii was observed in the presence of as low as 0.3125% of Tween 20 [Figure 1], the lowest among the tested solvents. Deducing from the results obtained, it is suggested that the maximum tolerable concentration (v/v) to be used for dissolving test samples would be 1.25% for DMSO, methanol and ethanol and 0.16% for Tween 20. Working at or below these maximum tolerable concentrations with the solvents in BSLT should not give false positive results in experimental outcomes.

$LC_{50}$ values, another toxicity indicator, were calculated for the solvents using Probit analysis[5] on a Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada). The $LC_{50}$ values for DMSO, methanol, ethanol and Tween 20 were 8.5%, 6.4%, 3.4% and 2.5% respectively [Table 1]. At these concentrations 50% of *A. salina* would be killed due to the toxic effect exerted the solvents. DMSO was found to have the lowest cytotoxicity effect against *A. salina* whereas, the cytotoxic effect of Tween 20 was shown to be the most pronounced among the tested solvents.

## CONCLUSION

Abiding to the maximum tolerable concentrations for the solvents found in the present study would ensure the experimental BSLT results to be genuinely due to the test materials, thus preventing false and distorted results from being reported. Therefore, the identification of the maximum end point for each solvent is crucial in order to prevent the solvents from exerting cytotoxic effects on *A. salina* in BSLT. Each of the universal solvents, such as DMSO, methanol, ethanol and Tween 20, has its own maximum tolerable concentration for *A. salina*. Therefore, the amount of the solvent to be used in sample materials should be considered before conducting any bioassay. This would ensure unbiased experimental results to be produced.

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