Annexure I

ANN Weights

IW

-0.808385836602885 2.24541024112469 1.90654932178142
0.609091982820408 2.34171822561739 0.965362069393803
2.98984726096865 2.05428655451522 -1.72960154597842
-0.595788170570710 0.0799012621870021 -3.20390219181210
-1.95583753487404 -2.17460293001043 -1.59325365311263
-3.50917106173062 0.241551762981286 -0.461614318762941
-2.01508195833517 0.367347436256357 2.52097909406800
2.21224711694103 -0.999948898114357 -2.07988703826076
-2.21920868179033 -2.13632503841014 2.09994247372763
2.07961168050576 0.151925522654230 -2.35172704564944
1.04838782256622 -1.85571847547438 2.25435384408603
-1.93372612177476 2.65414090388499 -0.174029290807205
-0.745568362041493 1.19156356665606 3.00520309362287

LW

-0.652325017331999 -0.0502175356076289 -0.661068277306957
1.21989290983133 0.193560828117106 -1.39890836283379 -
0.163399806260426 0.850870026630242 1.09523897826336 -0.841064908510957
0.107645555433050 -0.538981037407103 0.275364098992689

Bias I

3.71119225777683
-3.59469112578572
-2.23422741268519
1.43251106520335
0.885330610576988
-0.0914581199343325
-0.482414846726668
0.909787323850301
-0.714340306804695
2.23280049952377
1.73279827013194
-2.76081399507691
-3.26914036835649

Bias II

-0.208593523639734

GA constrains

Equality constraints

1. Addition should be equal to 1*x1+1*x2+x1*3=200
2. Acetone is always 70% of chloroform X1*0.7-x2*1 + x3*0=0

Bound Constraint matrix for Matlab
[98 68.6 183]
[10 7 33.34]

GA results
Optimum point

Chloroform (mL)	Acetone (mL)	Ethanol (mL)
63.05495690835494	44.13760376975034	92.80657325579655

Maximum Value: **6.47 mg/mL**

The Extra Code

```
function F = snl(x);
global sann t v
y=[x(1); x(2); x(3)]
n=sann(y); # sann is the neural network
if (t<n)
    t=n;  # Storing the value of maximum  recovery in a global variable
    v=x(1);  # Storing the value of chloroform in a global variable
    d=-n;
elseif (t>n)
    d=-n;
elseif (t==n)
        if(x(1)>=v)# rejecting any higher or equal chloroform volumes
                   resulting in same recovery
         n=n-1;
         d=-n;
        elseif (x(1)<v)
            v=x(1)
            d=-n;
        end
end
F=d;
end
```

GAoptions = Default otherwise mentioned

PopulationType: []
PopInitRange: []
PopulationSize: 250
EliteCount: []
CrossoverFraction: []
ParetoFraction: []
MigrationDirection: []
MigrationInterval: []
MigrationFraction: []

```
      Generations: 50
        TimeLimit: []
      FitnessLimit: []
     StallGenLimit: 10
    StallTimeLimit: []
           TolFun: []
           TolCon: []
 InitialPopulation: [100x3 double]
    InitialScores: [100x1 double]
   InitialPenalty: []
    PenaltyFactor: []
     PlotInterval: []
      CreationFcn: []
 FitnessScalingFcn: []
     SelectionFcn: []
     CrossoverFcn: @crossoverscattered
      MutationFcn: []
DistanceMeasureFcn: []
       HybridFcn: []
         Display: 'off'
        PlotFcns: {[@gaplotbestf]  [@gaplotbestindiv]}
      OutputFcns: []
      Vectorized: []
     UseParallel: []
```

All the [] options are at default values.

Annexure II

Constrained Azeotropic Optimization of Extraction System Components for Safe and Efficient Recovery of Betulinic acid

Medicinal Significance of Betulinic acid

Betulinic acid has wonderful pharmacological activities which makes this molecule commercially important.[1,2] Because of its selective cytotoxicity against tumor cells and favourable therapeutic mode of action, betulinic acid is a very promising newer chemotherapeutic agent for the treatment of cancer and HIV infections.[3] Betulinic acid is known to have thyroid-enhancing potential by lowering thyroid-stimulating hormone levels and reducing thyroid tissue damage, thereby minimizing the symptoms of hypothyroidism

when used anaphylactically in rats.[4] Our group and collaborators have published several research articles regarding fermentative recovery, extraction and antimicrobial properties of betulinic acid.[5,6] Some very interesting modelling and optimization studies for betulinic acid production from microbial bioconversion of betulin are under publication from our group using statistical and artificial neural network techniques and the current study is the extraction part of that unpublished data. Betulin is used as a precursor for the synthesis betulinic acid via bioconversion. Betulin transformations were commonly done by many microorganisms as biocatalyst.[7,8] In this study, we have successfully used suitable betulin catalysing bacteria for the maximum bioconversion of betulin to betulinic acid under optimum conditions (work to be published soon). In order to test our strategy we have extracted betulinic acid from this optimized fermentation broth using different solvents or solvent systems utilizing azeotropic distillation.

Below Mentioned Experiments Were Performed to Test the Suggested Strategy (given in the manuscript)

Comparison of Solvents or Solvent Systems (SS)

The fermentation broth (50 mL) recovered from the shake flask was subjected to microfiltration by a 0.22 μm ceramic cartridge in order to remove the microbial cell debris and other undissolved material. The clear culture filtrate containing betulinic acid was then subjected to extraction (1:1 v/v) under optimum process conditions (this is our unpublished data). All the azeotropic Solvent Systems (SS) mentioned in Table 1 (below) have azeotropic composition for each component of the system. For all the non-azeotrope SS mentioned in Table 1, the components of the system were maintained in equal proportions (1:1 for binary; B and 1:1:1 for ternary; T). SS No. 0-3 are pure individual solvents. Each SS was subjected to extraction of betulinic acid from the fermentation broth.

Table 1: Comparison of the solvent systems (SS)

SS No	Mix	Component 1		Component 2		Component 3		Azeotrope (if any)		Betulinic acid recovery
		Solvent	BP (°C)	Solvent	BP (°C)	Solvent	BP (°C)	Type	Az BP (°C)	(mg/L)
0	L	n-Hexane	68.95	-	-	-	-	Non-azeotrope	-	0.33±0.02
1	L	Methanol	64.5	-	-	-	-	Non-	-	0.28±0.03

								azeotrope		
2	L	Methyl acetate	56.3	-	-	-	-	Non-azeotrope	-	0.25±0.03
3	L	Ethyl acetate	77.1					Non-azeotrope		0.29±0.02
4	B	Methanol	64.5	Ethyl acetate	77.1			Minimum boiling	59.1	0.26±0.02
5	B	Methanol	64.5	Methyl acetate	56.3			Minimum boiling	53.9	0.24±0.02
6	B	Methanol	64.5	n-Hexane	68.95			Minimum boiling	49.5	0.3±0.02
7	T	n-Hexane	68.95	Ethyl acetate	77.1	Methanol	64.5	Non-azeotrope		0.3±0.03
8	T	n-Hexane	68.95	Methyl acetate	56.3	Methanol	64.5	Minimum boiling	45	0.31±0.02

Optimization of Test Solvent System (TSS)

Table 2 (given below) shows azeotropic Test Solvent System (TSS) for n-hexane, methanol and methyl acetate. Each TSS contained n-hexane and methanol in exact ternary azeotropic ratio while methyl acetate was maintained in excess of the ternary azeotropic ratio. Each TSS was of 200 mL only. The TSS No. 24-30 are same, this was done to minimize the degree of error. Each TSS was subjected to extraction under similar conditions. Methanol and methyl acetate present in TSS are quiet miscible with water, direct extraction of betulinic acid from aqueous fermentation broth with TSS will disturb the azeotropic composition of the TSS. Since, the extraction potential of n-hexane is maximum (Table 1), betulinic acid is primarily extracted with n-hexane (volume as per Table 2, per 200 mL of the fermentation broth) that forms a upper separate immiscible organic layer (containing betulinic acid) which is then collected separately and mixed with the corresponding azeotropic ratio of methanol and excess of methyl acetate (as mentioned in Table 2) forming a complex ternary azeotrope. The extracted yield of betulinic acid (column 5, Table 2) served as targets and volume of n-hexane, methanol and methyl acetate in column No. 2-4 of Table 2 served as inputs for training, validation and testing of the ANN. All the experiments were performed in triplicate and average are reported.

Table 2: Azeotropic Test Solvent System (*Tr*: Training TSS; *V*: Validation TSS; *T*: Testing

TSS. No	**n-Hexane (mL)**	**Methanol (mL)**	**Methyl Acetate (mL)**	**Observed Betulinic acid (mg/L)**	**ANN predictions (mg/L)**
1T	10.00	1.97	188.03	0.01	0.004667289
2Tr	14.00	2.75	183.25	0.012	0.01318799

3 T	18.00	3.54	178.46	0.016	0.026453249
4V	22.00	4.33	173.67	0.019	0.04513708
5 Tr	26.00	5.11	168.89	0.1	0.06811299
6 T	30.00	5.90	164.10	0.12	0.092944626
7 Tr	34.00	6.68	159.32	0.13	0.116423938
8 Tr	38.00	7.47	154.53	0.14	0.138969921
9 Tr	42.00	8.26	149.74	0.16	0.164742468
10 Tr	46.00	9.04	144.96	0.2	0.197697915
11 V	50.00	9.83	140.17	0.24	0.232812234
12 Tr	54.00	10.62	135.38	0.26	0.263124645
13 Tr	58.00	11.40	130.60	0.3	0.296930501
14T	62.00	12.19	125.81	0.32	0.344865305
15 Tr	66.00	12.98	121.02	0.33	0.334616267
16 V	70.00	13.76	116.24	0.34	0.344672599
17 Tr	74.00	14.55	111.45	0.3	0.311881779
18 V	78.00	15.33	106.67	0.29	0.29163476
19 Tr	82.00	16.12	101.88	0.3	0.293882356
20 Tr	86.00	16.91	97.09	0.3	0.299280479
21 Tr	90.00	17.69	92.31	0.31	0.302846552
22 Tr	94.00	18.48	87.52	0.3	0.304694874
23 T	98.00	19.27	82.73	0.3	0.305527758
24 Tr	54.00	10.62	135.38	0.26	0.263124645
25 T	54.00	10.62	135.38	0.28	0.263124645
26 V	54.00	10.62	135.38	0.29	0.263124645
27 Tr	54.00	10.62	135.38	0.26	0.263124645
28 Tr	54.00	10.62	135.38	0.24	0.263124645
29 T	54.00	10.62	135.38	0.25	0.263124645
30 Tr	54.00	10.62	135.38	0.25	0.263124645

Quantification of Betulinic acid

In order to quantify betulinic acid, all of the residual methyl acetate was evaporated in a rotavapor at temperatures above 60°C. The collected residue was redissolved in methanol and filtered through 0.22 µm millipore filter, then analysed by RP-HPLC (YL9112, South Korea). The HPLC system used for this study consists of Yong Ling's 9112 pump, YL9120UV-Vis detector, Prontosil C18 HQ105 H column (250 mm x 4.6 mm x 5 mm,). Samples were analysed under following condition: the flow rate was set at 1.0 ml/min under room temperature, mobile phase was composed of acetonitrile-water 91:09 (v: v). The wavelength was set at 210 nm.[9,10] To generate the calibration curve, betulin and betulinic acid were dissolved in dichloromethane diluted to various concentrations (25-100 µg and 10-50 µg/ml, respectively), and kept at 4°C in dark. Before the analysis, the solutions were filtered

with 0.22 μm Millipore filter and analyzed by HPLC (YL9112, South Korea). The calibration curve was constructed by plotting the peak area versus the ratio of their corresponding concentrations. The calibration curve showed good linearity between the peak area ratios against the concentration over the calibration ranges.

Artificial Neural Network Design and Training

Same as discussed in the manuscript (Kindly refer the manuscript).

Genetic Algorithm Based Optimization

Same as discussed in the manuscript (Kindly refer the manuscript).

Results

The extraction efficiency of each SS mentioned in Table 1 was revealed by its betulinic acid recovery. None of the SS appears to be better than pure n-hexane (SS No. 0). Hexane and methanol belongs to the restricted class 2 (as per the ICH guidelines). Solvents under class 3 (methyl acetate, acetone, ethanol etc.) are considered as less toxic and of lesser risk to human health. The ICH class 3 contains no solvent identified as a human health hazard at levels normally accepted in pharmaceutical preparations. Since, n-hexane was found to be the most suitable solvent for the extraction of betulinic acid, our objective was to determine the optimum volume of n-hexane for the extraction of betulinic acid and to finally obtain betulinic acid in a class 3 solvent in an energy efficient manner. This is achieved by making several combinations of a ternary azeotrope of hexane with methanol and methyl acetate. Each row of Table 2 forms a ternary azeotrope wherein all the n-hexane and methanol are in exact azeotropic ratio and methyl acetate is in excess. This solvent system carrying excess of entrainer (methyl acetate) when reaches the Az BP; during the downstream process (concentrating betulinic acid by evaporating the solvent at higher temperatures) all the n-hexane and methanol (both ICH restricted class 2 solvents) along with little methyl acetate (as per ternary azeotropic volumetric ratio) will distil out safely leaving excess of pure methyl acetate (ICH class 3 safe solvent) as residue. Since the residue contains only pure methyl acetate (≥99.5%) we can safely maintain betulinic acid at desired concentration levels (in methyl acetate). This method of downstream processing is quiet energy efficient since the boiling point of the ternary azeotrope considered in this study (45°C) is well below the boiling point of any of the individual solvents (Table 1). Hence, lesser energy will be required to safely distil out class 2 solvent (i.e., n-hexane and methanol).

Optimization of the solvent system and the application of constraints were done in the same manner as discussed in the principal manuscript. However, the components of the Test Solvent System were kept within the following respective range.

Component	*Lower Bound (mL)*	*Higher Bound (mL)*
n-Hexane	*10*	*98*
Methanol	*1.9*	*20*
Methyl acetate	*82*	*189*

The trained and validated feed-forward backprop network with ten hidden layers is shown in Figure 1. The performance of the network is shown in Figure 2. Regression coefficients for Training (Tr), Testing (T) and Validation (V) lie very close to 1 defining an adequately trained and validated network.

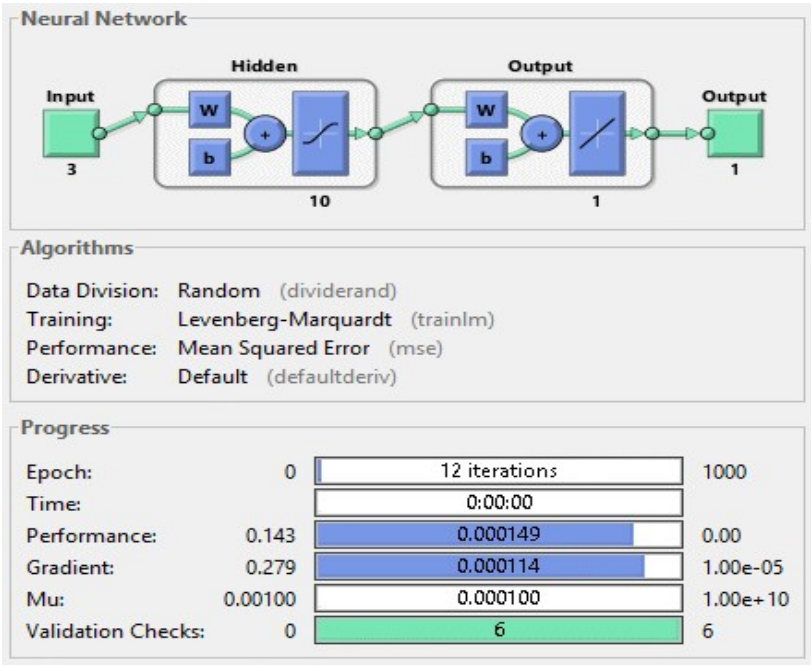

Figure 1: Trained and validated Artificial Neural Network

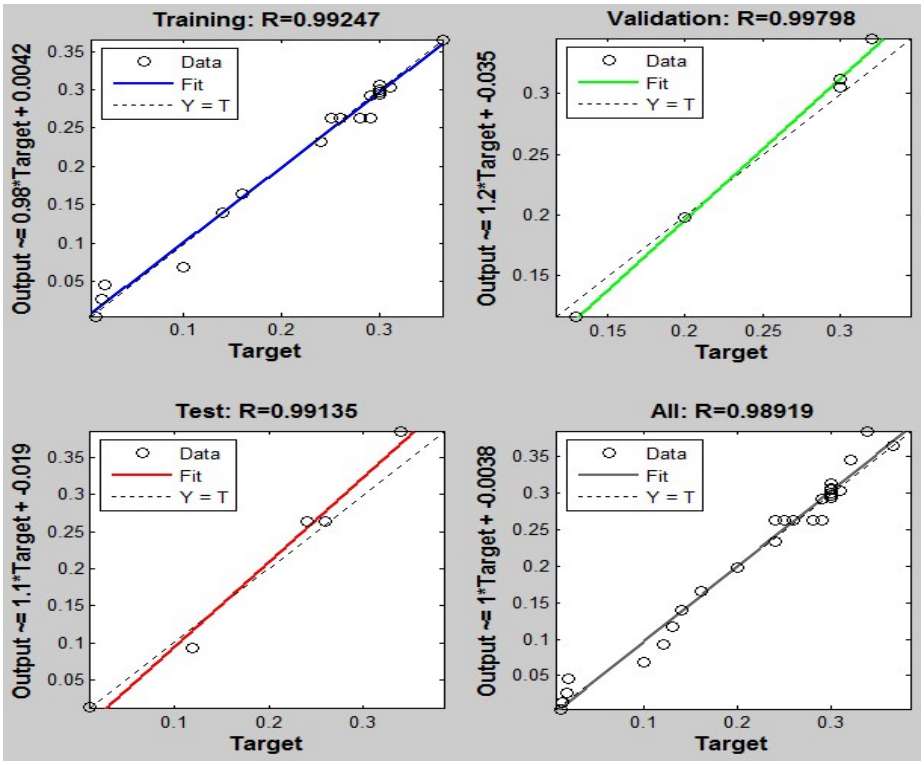

Figure 2: Training, Testing and validation Regression coefficients

Genetic algorithm (GA) optimization of the neural network was done in the same manner as mentioned in the manuscript. The constraints were applied using equality matrices and bounds. The optimum combination of the solvents resulting in maximum betulinic acid recovery is shown in Table 3 and Figure 3.

Table 3. Betulinic acid recovery in different solvents

	n-Hexane	**Methanol**	**Methyl acetate**	**Maximum Betulinic acid recovery (mg/L)**
GA prediction	66.9064240635182	13.15480095145560	119.9396634219262	0.386
Experimentally Observed	66.9	13.15	119.93	0.4±0.02

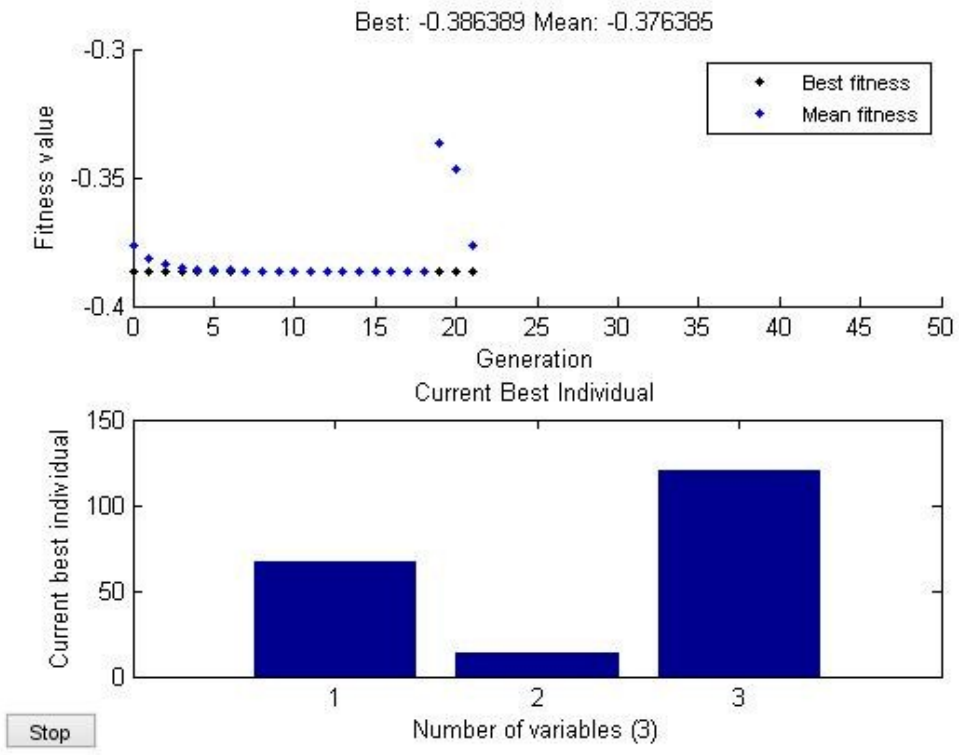

Figure 3: Genetic algorithm optimization

If the optimum volumetric azeotropic combination of n-hexane, methanol and methyl acetate is known, one can easily calculate the critical n-hexane free residual volume (V_{hr}) from the volumetric azeotropic ratio of n-hexane, methanol and methyl acetate in an azeotropic ternary complex. For safety and indemnity achieving the residual volume ≤ 70% of the calculated V_{hr} assures ~100% n-hexane and methanol free recovery (V_{hfr}: $Hexane\ and\ methanol\ free\ residual\ volume$) of the metabolite; in this case, i.e., betulinic acid:

$$V_{hr} = 200 - Hexane\ optimum\ volume\ (mL) - methanol\ optimum\ vol\ (V_m) \quad (1)$$

$$V_m = 0.3218\ hexane\ optimum\ volume\ (mL) \quad (2)$$

$$V_{hfr} = 0.7 V_{hr}$$

In this case, $V_{hfr} = 68.89\ mL$.

Finally reducing the volume of solvent system to 68.89 mL ensures complete n-hexane and methanol free recovery of betulinic acid. The residual volume V_{hfr} was subjected to GC/MS

analysis (TSQ 8000 Evo Triple Quadrupole GC-MS/MS; Thermofisher Scientific Inc.) for the determination of hexane and methanol. A negligible level of hexane and methanol was observed in the final free residual volume V_{hfr} (well below the prescribed ICH threshold).

References

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