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BIOPROSPECTION FOR IDENTIFICATION OF PROMISING ANTIMICROBIALS OF HERBAL ORIGIN AGAINST HIGHLY VIRULENT STRAINS OF INFLUENZA VIRUS

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ABSTRACT:

H5N1 is enzootic in many world populations especially in Southeast Asia. One strain of HPAI- A (H5N1) i.e. Highly Pathogenic Avian Influenza Type A, Subtype H5N1, is spreading globally after first appearing in Asia in between 2003 to 2011, a total of 566 confirmed human cases have been reported resulting in 332 deaths in several countries. Treatment with anti-influenza drugs, like amantadine or neuraminidase inhibitors, resulted in emergence of resistant variants. Therefore, further virological studies are urgently needed and effective strategic measures for new and amply available antiviral agents for mitigating avian influenza A (H5N1) virus must be desirable. The current bioprospective study aims to investigate the probable potential of various natural plants dependent on bioactivity parameters and presence of chemical constituents, using matrix based modeling, accompanied by optimization. The outcomes of the lead identification need verification for confirming antiviral potential against virulent H1N1 virus.

Keywords: *Antiviral agents, Herbal mitigation, Matrix modeling, Ethno pharmacology, Influenza Virus, H1N1*

[I] INTRODUCTION

The influenza virus, accepted as a circulating pathogen inside the population since 16th century, is notable to its unique capacity to cause recurrent epidemics and global pandemics [1]. The ability of the virus is to undergo genetic reassortments with unpredictable adjustments in its antigenic makeup leading to the consequent immunologic response thereby resulting in recurrent epidemics of febrile respiratory disorder every 1–36 months [2].

In the 20th century, three influenza pandemics occurred and killed lots of people, attributed to the emergence of a new virulent recombinant viral strain, transmitting to humans [3]. In April 2009, a novel flu strain evolved with a genetic reassortments of genes from human, pig, and bird flu, initially termed as 'swine influenza' or influenza A/H1N1, emerged in Mexico, United States, and lots of other nations leading to more than 1817 deaths till date [4].

Presently, two classes of antiviral drugs happen to be licensed by the US Food (FDA) for preventing influenza virus infections: M2 ion channel blockers (amantidine and rimantidine) impressive against influenza A viruses and neuraminidase inhibitors (oseltamivir - Tamiflu and zanamivir - Relenza), replacements against both type A and type B influenza infections [5]. The Neuraminidase inhibitors (NAIs) concentrate on the active site with the Neuraminidase (NA) protein, inhibiting its sialidase activity which is required for the release of the virion [6]. However, it is considered that the creation of antiviral resistance may limit the clinical utility of the drug down the road. With the advent of sturdy viral strains like H7N9, H5N1 etc., it has become essential to look for novel antivirals as alternative agents from medicinal plants, which could serve as sources of compounds that are useful in managing newly emerging viruses.

A multipronged approach is needed to spur alternative antimicrobial agent's development, with mechanisms to support current products, as well as encourage the development of new ones. Our previous study of evidence based alternatives provided a database of 50 herbals targeting various aspects of infection cycle and symptomatic care [2].

The present classical bioprospection approach is a systematic methodology adopted by utilizing *in silico* herbal bioprospection approach integrated with ethnopharmacological importance to screen against the identified flora of potential candidates. This model led to the selection of 09 potential herbal candidates against novel H1N1.

[II] MATERIALS AND METHODS

2.1. Selection of Microorganism

Rationale of selection of a targeted microorganism was based on acquisition of certain important characteristics including a) category of microorganism as lethal, sub-lethal, incapacitating or very damaging Biothreat agent; b) unavailability of a treatment regime/vaccine available or limited availability; c) re-emerging and recombinant virulent forms from past; d) possibility of its use as a bioweapon which might be lethal and/or panic creating agent.

2.2 Selection of Bioactivity Parameters using Classical Approach

Based on the understanding of the mechanistic aspects of Influenza virus infection and epidemiology, as in present study, H1N1 virus, various virulence factors were targeted on the basis of extensive literature surge (Classical Bioprospection Approach).

Also, the herbal plants were selected for the study on the basis of their indirect or direct potential of treating dreadful infections allied with virulent viral strains, like H1N1 Virus. The seven testing parameters were selected for study based on mechanistic aspects of H1N1 virus strains, including (a) Neuraminidase inhibition, (b) Hemagglutinin inhibition, (c) Immunomodulation, (d) Interferon inhibition, (e) Transcription inhibition; (f) Symptomatic relief provision and (g) Presence of phytocompounds [7]. The rationale supporting selection of these parameters using classical approach for bioprospection studies are given in Table 1.

2.3 Evaluation of Relevance Factor using Keywords Hits Scoring Matrix Approach

The analysis was executed using PubMed as selected search engine. The random search model using combination keyword as Bioactivity Parameter + Antimicrobial activity while advanced search model using the same combination keywords but in quotes, yielded 'N' hits. The first n=20 hits were provided by the search engine, working on the principle of priority indexing. The first 20 hits are subjected to observational interpretation for assessing relevance using human interface. This sample set based analysis was used to evaluate the net weightage linked to each bioactivity parameter, using the following formula (Eqn. 1);

Average Percentage Relevance = $\frac{(\text{No.of Relevant hits based on observational analysis} \div N)}{(n=20)} X\,100$

Relative weightage for each parameter was assigned on the basis of percentage relevance as given in Table 2.

2.4 Selection of Herbal Plants using Classical Bioprospection Approach

The classical bioprospection approach accounts for investigation of the following variables based on literature review to devise a logical conclusion, resultant in selection of plants. It includes a) Ethnopharmacological importance of plant; b) Relevance of Herb in traditional medicine; c) Availability factor or cultural acceptability in localized regions; d) Any vedic literature supporting its use; e) Investigations/ prior experience on potential of the herb; f) Indirect indications, if any etc. The final conclusion to select a plant for in silico bioprospection is based on learning of the subject area conjugating with prior experiences/ investigations. The rationale for selected plants is given in Table 3.

2.5 Binary Coefficients Matrix to Evaluate the Presence/Absence of a parameter in selected plants-This methodology works on the principle of 0-1 binary code of absence/presence of a particular parameter in selected plants from previous step. The range of outcome of matrix lies between 1 to 8 for any plant in the present case. The cut off value selected for this matrix based analysis is closest value to the median of 1-8 range. Based on this, all the plants having more than 03 parameters, reported in PubMed search engine (n= first 20 hits) against 'Bioactivity Parameter + Selected Plant' random search model, were selected. It relates to the fact that only these plants which can support holistic approach should be screened for the next level analysis, in line with the rationale of present study.

2.6 Weightage Matrix Based Analysis

This step includes evaluation of overall weightage of plants (Scores > 3 in previous step) by multiplying their binary score with weightage obtained in Step No. 2.5 [8]. This is a primary step to screen the plants utilizable to subsequent analysis and removes false positives attributed towards investigator's biasness due to 'experience factor'. This step enhances the 'uncertainty factor' required for obtaining statistically valuable outcome. This step identifies potential plant leads based on in silico bioprospection approach subjected to fuzzy set membership analysis and optimization to validate the findings. Weightage matrix score for the selected herbal plants is exemplified in Table 4.

2.7 Fuzzy Set Membership Analysis for Decision Matrix

In this approach, the given mathematical relationship was used to calculate the relevance of the variety/product (Eqn. 2);

$\mu S = S\text{-min}(S)/[\max(S)\text{-min}(S)]$

where, μS represents the desirability values of members of the fuzzy set S. Min(S) and max(S) are minimum and maximum values, respectively, in the fuzzy set S [9]. Scores after fuzzy set membership analysis of selected herbal plants are represented in Table 4.

2.8 Optimization of Decision Matrix Score

In this approach the numerical value of scores obtained were converted into a leveled score by using a scaled magnitude represented by a symbol.

[III] RESULTS

3.1 Keywords Hits Scoring Matrix

On the basis of the keyword hits scoring results weightage was given to various parameters selected for screening of herbal plants with respect to antimicrobial activity. Weightage was decided according to the percentage relevance obtained for each parameter, as elucidated in table 2. Highest percentage relevance was obtained for transcription inhibition, followed by other parameters like immunomodulation, interferon inhibition, neuraminidase inhibition, hemagglutinin inhibition, symptomatic relief provision and presence of phytocompounds. Consequently weightage factors were given to selected parameters in the range of 1-4, based on statistical unitary approach, with highest weightage i.e. 3.4, given to transcription inhibition, followed by other parameters in decreasing order, as explicated in Table 4.

3.2 Binary (Presence-Absence) Coefficients Matrix

Out of 20 herbal plants, 9 herbal plants were shown to contain more than 3 characteristic and hence illustrated a score higher than cut off as compared to other herbs, e.g. *Glycyrrhiza glabra, Ocimum sanctum, Alium sativum, Zingiber officinale, Azadirachta indica, Camellia sinensis, Illicium verum, Ginkgo biloba and Olea europaea*, as shown in figure 1.

3.3 Simple Additive Weighing Matrix followed by Decision Matrix

Out of 9 plants selected on the basis of binary coefficient matrix (Binary Matrix score > 3), it was revealed that 6 herbal plants showed immense potential of acting as a therapeutic agent against H1N1 virus, as their combined weightage scores were even higher than the median value score i.e. 8.6, e.g. *Glycyrrhiza glabra, Ocimum sanctum, Zingiber officinale, Illicium verum, Ginkgo biloba and Olea europaea*, as shown in Table 4.

3.4 Optimized Scoring

Optimized values were given to the selected herbal plants, on the basis of which top 6 relevant herbal plants were revealed, e.g., *Glycyrrhiza glabra, Ocimum sanctum, Zingiber officinale, Illicium verum, Ginkgo biloba and Olea europaea*, as shown in Table 4.

[IV] DISCUSSION

Antivirals have become the mainstay of our lifestyle disease control strategy as these chemotherapeutic agents are often used to treat many infectious viral diseases. An overuse and abuse of such agents has led to the transformation of the sensitive viral strains into mutant ones through natural selection, high frequency recombinations and defined mutations. An alternative pipeline of herbal / natural therapeutic agents has to be searched for the adequate control of the emergence, re-emergence and spread of genetically sturdy microorganisms like H1N1 virus, showing recurrent genetic reassortments patterns.

The present study is an attempt to combine classical literature based analysis with statistical interpretation of data output obtained by both random and advanced search model using the PubMed web tool. The seven parameters were selected on the basis of their direct significance in a) symptomatic relief provision, b) viral replication inhibition, c) immunomodulatory potentials, (Table-1). Similar study using web tools for bioprospection has been done by Elizabeth S. Jenuwine and coworkers for the evaluation of sleep wake cycle in healthy individuals (selected parameter) [10]. This similar model using multi-parametric approach has been used in conjunction with evaluation of their relative relevance based on priority indexing (Table-2).

In this study, selection of plants has been done on the basis of parameters like ethnopharmacological importance, use in traditional medicine, ease of availability, any indication in Vedic literature or available scientific evidence for complimentary use. Such investigatory analysis has provided 20 plants showing variable significance with respect to different descriptors chosen.

The plants were subjected to both random and advanced search model, (Using PubMed as search engine) followed by Binary Coefficient matrix analysis ($p < 0.05$), revealing 9 out of 20 plants to be used for in silico cross matrix bioprospection analysis. Binary coefficient matrix analysis is used to extract items of choice with probable higher significance based on all or none principle. This practice removes bulk outliers, thereby reduces database size to measurable proportion. This is achieved by selecting plants with binary score > 3, so as to scale down the number of plants from 20 to 9 and thereby reducing the timeframe of screening significantly (Figure 1).

The selected 9 plants are subjected to *in silico* bioprospection model where additive weightage matrix based analysis is performed to analyze the weighted scores for each selected herbal plant on the basis of presence of each weighted parameter. This matrix works on two principles a) binary (0/1) presence or absence law and b) weightage scoring analysis in conjunction. The net scoring was analysed in priority ranking providing relevance of natural plants with respect to their possible role in overall antiviral activity against H1N1 virus (Table 4). The optimization of obtained data was a fuzzy set membership analysis for decision matrix, revealing 06 plants with optimization score > 3.

[IV] CONCLUSION

As resistance to old antibiotics and antivirals spreads, the development of new antimicrobial agents has to be advanced if the problem is to be contained. Bio-assay would reveal the presence of multiple antimicrobial compounds or synergistic effects of these compounds. Therefore, standardization of active fractions and study for toxicity and *in vivo* efficacy may result in development of better antimicrobial drugs. It may provide nature friendly and cheap drugs accessible to all the people of world.

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Conflict of Interest: The authors declare no conflict of interests and strongly advocate that self-medication be avoided.

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Table 1. Rationale for Selection of the Bioactivity parameters for Bioprospection Study

S.No.	Parameter	Rationale for selection (Based on Classical Approach)
1	**Neuraminidase inhibitor**	a) These compounds showed activity against a wide variety of influenza strains, inhibited viral replication in cell culture, and were able to protect mice against influenza infection. Moreover, the compounds showed activity against drug-resistant strains in vitro e.g. zanamivir (Relenza) and oseltamivir (Tamiflu) b) Some plants have been reported to exhibit neuraminidase inhibition activity and are effective antimicrobial agents, e.g., *Illicium verum, Ginkgo biloba.* c) Neuraminidase catalyzes the removal of sialic acids from the surface of host cells to initiate entry. Discovery of a Neuraminidase–sialic acid intermediate led to the production of sialic acid analogs that bound covalently to NA and inhibited its enzymatic activity.
2	**Hemagglutinin inhibitor**	a) Influenza virus particles have an envelope protein called the hemagglutinin, or HA, which binds to sialic acid receptors on cells. b) Avian viruses which can agglutinate avian RBCs (hemagglutination) include NDV, Influenza, and ADENO 127. In addition to viruses, Mycoplasmas are also capable of hemagglutinating avian RBCs. Antibodies directed against these organisms will inhibit hemagglutination. c) Herbals namely *Ocimum sanctum, Camellia sinensis* have been found to show hemagglutinin inhibitory effects.
3	**Immunomodulation**	a) Immunomodulation is terms defined as the “treatment of disease by inducing, enhancing, or suppressing an immune response”. b) Cytokines, corticosteroids, macrolides, statins, diabetic medications (metformin, pioglitazone), fibrates, and cox-2 inhibitors are likely candidates for immunomodulation in H1N1. c) Plants such as *Glycyrrhiza glabra, Ocimum sanctum, Alium sativum, Zingiber officinale, Phylanthus emblica, Tinospora cardifolia, Azadiracta indica, Aegel marmalose, Tachyspermum ammi, Illicium verum,etc* showed immunomodulatory effects.
4	**Interferon inhibitor**	a) Many viruses modulate the interferon signaling pathway by inhibiting the cellular proteins participating in this cascade. They can interfere with basically all the components participating in the expression of interferon-stimulated genes. The type I interferon (IFN) response represents one of the first lines of defense against influenza virus infections. b) Both IFN-alpha and IFN-beta inhibited the replication of the pandemic (H1N1) virus. The potential of IFN-lambda3 to inhibit viral replication was lower than that of type I IFNs. c) The novel pandemic (H1N1) influenza A virus can readily replicate in human primary DCs and macrophages and efficiently avoid the activation of innate antiviral responses. It is, however, highly sensitive to the antiviral actions of IFNs, which may provide us an additional means to treat severe cases of infection especially if significant drug resistance emerges. Plants such as *Illicium verum, Glycyrrhiza glabra, Ginkgo biloba, Olea europaea* etc show interferon inhibitory effects.

5	**Nuclear receptors**	a) Nuclear hormone receptor proteins form a class of ligand activated proteins that, when bound to specific sequences of DNA serve as on-off switches for transcription within the cell nucleus b) Increased NF-êB activation, as well as inhibition of the negative regulator TRIM24, early and persistent infiltration of immune cells, including inflammatory macrophages, and the absence of activation of lipid metabolism later in infection, which may be mediated by inhibition of nuclear receptors, including PPARG and HNF1A and -4A. c) Transmission of the H1N1 influenza viruses was further corroborated by characterizing the binding specificity of the viral hemagglutinin to the sialylated glycan receptors (in the human host).Plants such as *Zingiber officinale, Ocimum sanctum, Illicium verum* has been shown to exhibit similar mechanisms.
6	**Symptomatic relief provided**	a) For swine flu natural and herbal remedies are better treatment options, and they are free of side effects. Ayurvedic medicines and plant based medicines are being used for swine flu; are safe for human beings. More than 700 plants like Ginger (*Zingiber officinale*) and Holy Basil (*Ocimum sanctum*) etc are being used for many types of disease including swine flu. These plants have lot of antibiotic properties. b) This broadens the scope of our study related to H1N1 spread, and selection of plants on the basis of symptoms caused. c) The main symptoms are fever (101-103°F) with chills or shivering, and headache last up to a week, are similar to those of seasonal flu, and can include fever, sneezing, sore throat, cough, headache, and muscle or joint pain. With predefined vulnerability of patient showing these symptoms herbal agents can be utilized to prevent aggravation of infection.
7	**Presence of phytocompounds**	a) Presences of phytocompounds such as polyphenols, flavonoids, saponins, glucosides, and alkaloids etc have antiviral combating, immunostimulatory activity. b) Approach requiring Potential herbs that have been evaluated for their efficacy against flu viruses and have the above phytocompounds may be helpful in combating H1N1 pandemic. c) Herbal plants can be selected on the basis of presence of phytocompounds. Thus, alternative therapeutic agents should include diverse phytochemicals enriched herbals for holistic mitigation.

Table 2. Weightage assigned to the parameters based on Average Percentage Relevance

S.No.	Parameter Chosen	Relevant Hits with "Bioactivity Parameter + Antiviral activity" as keyword	Articles screened*	Total number of Hits	%age Relevance Index (P_0)	Optimized Value (%age relevance)	Weightage
1	Neuraminidase Inhibitor	14	20	149	(14/20)X1OO =70%	70% (149)	2.8
2	Hemagglutinin Inhibitor	13	20	51	(13/20)X100 =65%	65% (51)	2.6
3	Immunomodulatio n	16	20	484	(16/20)x100 =80%	80% (484)	3.2
4	Transcription Inhibitor	17	20	430	(17/20)x100 =85%	85% (430)	3.4
5	Interferon Inhibitor	15	20	926	(15/20)x100 =75%	75% (926)	3
6	Symptomatic Relief Provider	7	20	65	(7/20) X 100 = 35%	35% (65)	1.4
7	Presence of Phytocompounds	5	20	808	(5/20) X 100 = 25%	25% (808)	1

Table 3. Selected Herbal plants showing probable utility against H1N1 Infection

S.No.	Herbal Plant	Ethnopharmacological Importance	Availability	Vedic Literature supporting its use	Prior investigation	Indirect Indications (if any)
1	*Glycirrhiza glabra*	Used to treat upper respiratory track ailments including cough, hoarseness, sore throat bronchitis also in digestive ailments, ulcers, as a laxative.	Native to India and some Asian countries.	Reported in both Ayurveda and Greek Medicine and folk medicine.	Anti-inflammatory, sore throat curing & antiviral, duodenal ulcers curing effects have been already verified sub-clinically	Symptomatic relief in case of common cold, avian influenza and swine flu associated infections
2	*Ocimum sanctum*	Treatment of bronchitis, bronchial asthma, malaria, diarrhea, dysentery, skin diseases, arthritis, chronic fever, insect bite etc.	Originated in India and native to India.	Reported in Ayurvedic and folk medicine.	Anti-inflammatory, skin infection cure, headaches and eye disorders	Symptomatic relief in case of cold and flu, malaria, fever, eye infections and headaches.
3	*Allium sativum*	Asthma, Antibacterial usage and Chronic Bronchitis	Native to Asia but found all over the world.	Mentioned in The Bible, Vedas and Purans.	-	Symptomatic relief in case of asthma and other lung infections, antiseptic nature, anti-cancerous properties
4	*Zingiber officinale*	Detected for anti-rhino viral activities, Anti-motion, anti-inflammatory, anti-nauseate, hypolipidemic, anti-bacterial.	Indigenous to South-East Asia, cultivated all over the world.	Recorded in Ayurvedic, folk and Traditional Chinese Medicine.	Anti-inflammatory, analgesic, antipyretic, antimicrobial and hypoglycemic activities.	Symptomatic relief in case of nausea and is beneficial in treating upset stomachs. It is also anti-inflammatory and anti- viral.
5	*Azadirachta indica*	Abortifacient, analgesic, anthelminthic, antibacterial and antiviral	A native to east India and Burma.	One of the most recognized Ayurvedic herb. Antibacterial, anthelminthic, promotes oral health.	Anti-malarial, anti-cancerous, antithreupatic and cytotoxic.	Symptomatic relief in case of skin infections, malaria, antifungal and anthelminthic, fever and viral infections.
6	*Camellia sinensis*	Promotes secretion of gastrointestinal juices, lipolysis and glycolysis. Anti fatigue, Urine retention	Indigenously Cultivated in China, now grown in India and worldwide as a tea plant	Mentioned in Ayurveda and Chinese medicine	Anti-cancerous, anti-oxidant, anti-inflammatory, prevents dental caries. Antiviral, antibacterial	-
7	*Olea europaea*	Antiseptic astringent, hair tonic, ointment for rheumatic problems, nervous tension, high cholesterol, Antifungal, Antiviral, Antibacterial	Mediterranean region, Africa and Asia.	Reported in both Bible and Quran. Also used in Mediterranean folk medicine.	Anti-cancerous, Antifungal, Antibacterial, hair tonic, Antioxidant	Used in herpes, influenza, Epstein-Barr virus and HIV
8	*Ginkgo biloba*	Extract is helpful in memory loss, headache, ringing in the ears, vertigo, difficulty concentrating, mood disturbances, and hearing disorders.	Exclusively produced in china's eastern province.	Reported in Chinese medicine.	Studies show that extracts are mixed type inhibitors.	Symptomatic relief in memory loss, dizziness, Immunomodulation, bronchitis and asthma
9	*Illicium verum*	Used in actions of dispelling cold, analgesic and antimicrobial.	Native to Northeast Vietnam and China	Reported in Chinese medicine.	Shikimic acid produced have anti-influenza effects.	Antibacterial, carminative, diuretic, odontalgic.

Table 4. Fuzzy Set Membership Analysis for herbal plants screened on the basis of Weightage Matrix scores

S.No.	*Herbal Plant*	*Weighted Score*	*µS Score*	*Optimization Score*
1	*Glycyrrhiza glabra*	12	0.77	+++++
2	*Ocimum sanctum*	11.6	0.74	+++++
3	*Alium sativum*	8.6	0.5	++++

4	*Cocos nucifera*	5.4	0.24	+++
5	*Zingiber officinale*	12	0.77	+++++
6	*Phyllanthus emblica*	4.2	0.14	++
7	*Tinospora cordifolia*	5.6	0.26	+++
8	*Mentha piperita*	2.4	0	-
9	*Azadirachta indica*	9	0.53	++++
10	*Aegle marmelos*	5.6	0.26	+++
11	*Trachyspermum ammi*	5.6	0.26	+++
12	*Andrographis paniculata*	2.4	0	-
13	*Terminalia chebula*	5.6	0.26	+++
14	*Camellia sinensis*	9.2	0.54	++++
15	*Illicium verum*	14.8	1	++++++
16	*Ginkgo biloba*	11.4	0.72	+++++
17	*Olea europaea*	8.6	0.72	+++++
18	*Cinnamomum verum*	2.4	0	-
19	*Chrysanthemum indicum*	5.6	0.26	+++
20	*Withania somnifera*	5.6	0.26	+++

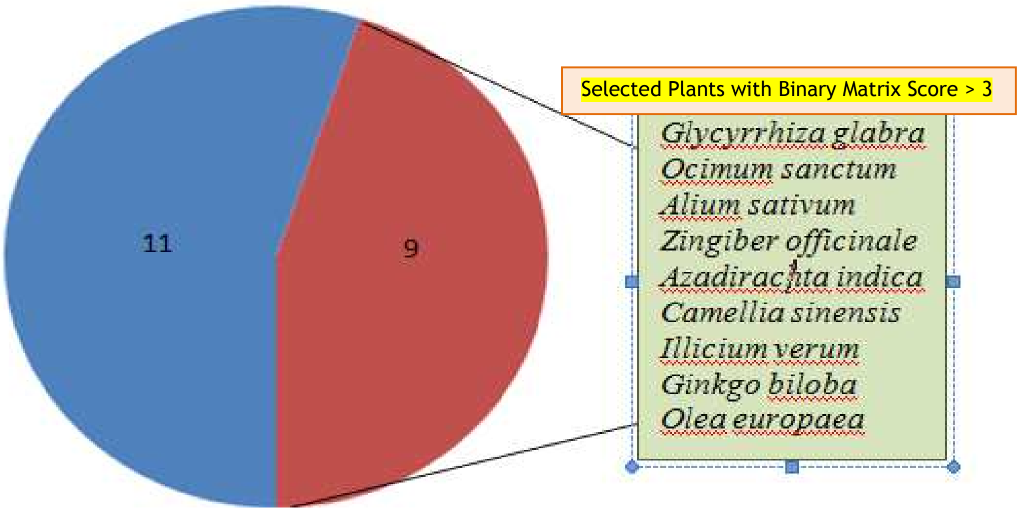

Figure 1. Binary Matrix Scores for Herbal Plants

Out of the 20 Plants studied, 09 plants had a binary matrix score of > 3 (*Glycyrrhiza glabra, Ocimum sanctum, Alium sativum, Zingiber officinale, Azadirachta indica, Camellia sinensis, Illicium verum, Ginkgo biloba* and *Olea europaea*) and rest 11 plants had a lower binary score of < 3 (*Coccos nucifera, Phyllanthus emblica, Tinospora cordifolia, Mentha piperita, Aegle marmelos, Andrographis paniculata, Terminalia chebula, Cinnamomum verum, Trachyspermum ammi, Chrysanthemum indicum, Withania somnifera*)